OPPTS HARMONIZED TEST GUIDELINES

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Health Effects

Volume I of III

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August 1998

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Office of Prevention, Pesticides, and Toxic Substances
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Series 870—Health Effects Test Guidelines

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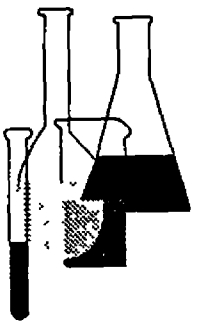
Health Effects Test Guidelines OPPTS 870.1000 Acute Toxicity Testing—Background



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.1000 Acute toxicity testing—background.

(a) **Scope—(1) Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) [Reserved]

(b) **Purpose.** The Agency considers the evaluation of toxicity following short term exposure to a chemical to be an integral step in the assessment of its toxic potential under the regulatory framework of its pesticide and toxic substances programs In the assessment and evaluation of the toxic characteristics of a substance, acute toxicity is generally performed by the probable route of exposure in order to provide information on health hazards likely to arise from short-term exposure by that route For pesticides, the short-term toxicity testing battery consists of acute toxicity tests by the oral, dermal, and inhalation routes, skin and eye irritation testing; and testing for dermal sensitization Data from an acute study may serve as a basis for hazard categorization, labeling, or child-resistant packaging and may also serve to designate pesticides which may be applied only by certified applicators It is also an initial step in establishing a dosage regimen in subchronic and other studies and may provide information on absorption and the mode of toxic action of a substance An evaluation of acute toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

(c) **History—(1) Acute toxicity test guidelines.** Test guidelines for acute toxicity were first published by the Agency in October 1982 as part of Subdivision F of the Pesticide Assessment Guidelines for the Office of Pesticide Programs (OPP) (see paragraph (f)(4) of this guideline) and in 40 CFR part 797 in September 1985 for the Office of Toxic Substances (OPPTS).

(2) **Rejection rate analysis.** In 1993, as part of its Pesticide Rejection Rate Analysis, Agency and industry scientists met to perform a guideline-by-guideline review of toxicology studies including acute toxicity studies The purpose of this guideline-by-guideline review was to identify those factors that most frequently cause toxicology studies required for pesticide reregistration to be rejected The results were published as the *Pesticide Reregistration Rejection Rate Analysis Toxicology* (see paragraph (f)(5) of this guideline) In 1995, representatives from the Agency met with the American Crop Protection Association (ACPA), the Chemical Producers and Distributors Association (CPDA), the Chemical Manufacturers Association (CMA), Health Canada, and the California Department of Pesticide Regulation (CDPR) to discuss acceptable methods for the conduct of acute

toxicity studies The discussions of this meeting were incorporated into a preliminary Registration Division document titled *Conduct of Acute Toxicity Studies* (see paragraph (f)(6) of this guideline) These documents supplement the acute toxicology guidelines in Subdivision F

(3) **Guideline harmonization.** The Series 870 Health Effects test guidelines have been harmonized between OPP and OPPTS and, where possible, with OECD test guidelines Scientific considerations from both of the analyses described in paragraph (c)(2) of this guideline have been incorporated into the revised test guidelines

(d) **Approaches to the determination of acute toxicity.** (1) At present, the evaluation of chemicals for acute toxicity is necessary for the protection of public health and the environment The Agency supports measures dedicated to reducing the use of animals in toxicity testing When animal testing is required for this purpose, testing should be done in ways that minimize numbers of animals used and that take full account of their welfare. To this end, when conducting a test, the Agency stresses the simultaneous monitoring of several endpoints of toxicity in animals in a single acute study including sublethal effects as well as lethality. Dosed animals are observed for abnormal behavioral manifestations such as increased salivation or muscular incoordination, in addition to the recovery from these effects during the observation period Both dead and surviving animals are necropsied to evaluate gross anatomical evidence of organ toxicity In selected cases, additional testing may be justified to better characterize the kinds of abnormalities that have been found in the organs of the necropsied animals These sound, scientific practices represent some of the means which maximize the utility of the data obtained from a limited number of test animals to achieve a balance between protecting humans and the environment, and the welfare and utilization of laboratory animals

(2) EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgements about safety

(i) Use of data from structurally related substances or mixtures In order to minimize the need for animal testing for acute effects, the Agency encourages the review of existing acute-toxicity information on chemical substances that are structurally related to the agent under investigation In certain cases, it may be possible to obtain enough information to make preliminary hazard evaluations that may reduce the need for further animal testing for acute effects Similarly, mixtures or formulated products that are substantially similar to well-characterized mixtures or products may not need additional testing if there are sufficient bridging data available for meaningful extrapolation In those cases, classification would be extrapolated from the mixture already tested

(ii) Use of appropriate alternative test protocols when available Thus, for example, acute oral toxicity testing may be performed using the Fixed Dose Method (OECD Guideline 420, see paragraph (f)(1) of this guideline), or the Acute Toxic Class Method (OECD Guideline 423, see paragraph (f)(2) of this guideline), or the Up-and-Down Method (OECD Guideline 425, see paragraph (f)(3) of this guideline) Abbreviated methods are not yet available through OECD for acute toxicity by other routes of exposure

(iii) Weight of evidence approaches to dermal and ocular irritation Several factors should be considered in determining the corrosion and irritation potential of chemicals before testing is undertaken Existing human experience and data and animal observations and data should be the first line of analysis, as it gives information directly referable to effects on the skin In some cases, enough information may be available from structurally related compounds to make classification decisions. Likewise, pH extremes (pH <2 or >11 5) may indicate dermal effects, especially when buffering capacity is known, although the correlation is not perfect Generally, such agents are expected to produce significant effects on the skin. It also stands to reason that if a chemical is extremely toxic by the dermal route, a dermal irritation/corrosion study may not be needed Likewise, if there is a lack of any dermal reaction at the limit dose (2,000 mg/kg) in an acute toxicity study (for which observations of dermal reactions were made), a dermal irritation/corrosion study again may not be needed. It should be noted, however, that often acute dermal toxicity and dermal irritation/corrosion testing are performed in different species that may differ in sensitivity *In vitro* alternatives that have been validated and accepted may also be used to help make classification decisions

(iv) All of the available information on a chemical should be used in determining the need for *in vivo* dermal irritation testing. Although information might be gained from the evaluation of single parameters within a tier (e g , caustic alkalies and acids with extreme pH (pH <2 or >11.5) should be considered as dermal corrosives), there is merit in considering the totality of existing information and making an overall weight of evidence determination. This is especially true when there is information available on some but not all parameters

(v) Use of limit testing For chemicals judged to be relatively nontoxic, a single group of animals is given a large dose of the agent. If no lethality is demonstrated, no further testing is pursued The substance is classified in hazard categories according to the limit dose used (See the following paragraph for a discussion of toxicity categories under FIFRA)

(e) **Regulatory applications under FIFRA.** (1) Precautionary labeling provides the pesticide user with a general idea of the potential toxicity, irritation and sensitization hazard associated with the use of a pesticide

(see EPA Label Review Manual (paragraph (f)(7) of this guideline) and 40 CFR Part 156—Labeling Requirements for Pesticides and Devices) Precautionary labeling also identifies the precautions necessary to avoid exposure as well as any personal protective equipment which should be used when handling a pesticide and statements of practical treatment in case of accidental exposure The United States is an active participant in negotiations to develop a globally harmonized system for classification and labeling. Planning for the globally harmonized system will be completed in the year 2000 with implementation to be phased in after planning is completed This section describes the current system in place for pesticides in the United States and will be revised and updated when the globally harmonized system is fully implemented.

(2) Precautionary labeling which includes the signal word, personal protective equipment, hazard symbol, and statements of practical treatment is normally determined by six acute toxicity studies and product composition. The acute oral, acute dermal and acute inhalation studies are used to determine the LD_{50} of a product via the designated route of exposure The primary eye irritation and primary skin irritation studies measure the severity of irritation or corrosivity caused by a product The dermal sensitization study determines whether a product is capable of causing an allergic reaction With the exception of the dermal sensitization study, each acute toxicity study is assigned a toxicity category as defined in the table below All products falling into toxicity categories I–IV must bear a signal word and in some cases warning symbols

(3) Personal Protective Equipment Personal protective equipment which includes use of protective clothing, chemical resistant gloves, protective eye gear, and respiratory protective devices, is determined by the results of six acute toxicity studies according to toxicity category (see table). The degree of protection required is graded according to the degree of acute toxicity and the hazard classification category of the chemical or product These requirements are set forth in 40 CFR 170 240 in the Worker Protection Standard

(4) Restricted entry intervals Agricultural products must display a restricted entry interval A restricted entry interval is the time immediately following a pesticide application during which entry into the treated area is restricted. Restricted entry intervals are based on the most severe acute toxicity category assigned to the acute dermal, eye irritation and skin irritation data for all of the active ingredients in a pesticide product. The duration of restricted entry intervals is based on the severity of toxicity, with products classified in category I requiring intervals of 48 hours or more and products classified in category III or IV requiring intervals of 12 hours

(5) Child-resistant packaging FIFRA establishes standards with respect to pesticide packaging of products intended for use in residential

settings in order to protect children or adults from serious illness or injury resulting from accidental ingestion or contact with pesticides Criteria for which pesticides must be distributed or sold in child-resistant packaging are based on classification according to the toxicity categories set forth in the table

(6) Restricted use pesticide The Agency determines whether a pesticide must be applied under the direct supervision of a certified applicator Such clarification for restricted use is based upon consideration of toxicity data, including acute toxicity, exposure, and intended use

(7) Biochemical pest control agents are tested in a special tiered progression The technical grade biochemical pest control agent is always characterized by acute toxicity tests However, because of their nontoxic mode of action against the target pest, further testing of the biochemical pest control agent is normally not required Microbial pest control agents are tested using the OPPTS Harmonized Test Guidelines Series 885, Microbial Pesticide Test Guidelines, for pathogenicity/infectivity In addition, all formulations of microbial pest control agents are tested for precautionary labeling using acute toxicity tests in the OPPTS Harmonized Test Guidelines Series 870, Health Effects Test Guidelines

Toxicity Categories

Study	Categoryl	Category II	Category III	Category IV
Acute Oral	Up to and including 50 mg/kg	>50 through 500 mg/kg	>500 through 5000 mg/kg	>5000 mg/kg
Acute Dermal	Up to and including 200 mg/kg	>200 through 2000 mg/kg	>2000 through 5000 mg/kg	>5000 mg/kg
Acute Inhalation	Up to and including 0 05 mg/liter	>0 05 through 0 5 mg/liter	>0 5 through 2 mg/liter	>2 mg/liter
Eye Irritation	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days	Corneal involvement or irritation clearing in 8-21 days	Corneal involvement or irritation clearing in 7 days or less	Minimal effects clearing in less than 24 hours
Skin irritation	Corrosive (tissue destruction into the dermis and/or scarring)	Severe irritation at 72 hours (severe erythema or edema)	Moderate irritation at 72 hours (moderate erythema)	Mild or slight irritation (no irritation or slight erythema)

Study	Study results	Study results
Dermal Sensitization	Product is a sensitizer or is positive for sensitization	Product is not a sensitizer or is negative for sensitization

(f) **References.** The following references should be consulted for additional background information on this test guideline

(1) Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals Guideline 420 Acute Oral Toxicity-Fixed Done Method Adopted July 17, 1992.

(2) Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals Guideline 423· Acute Oral Toxicity-Acute Toxic Class Method Adopted March 22, 1996

(3) Organization for Economic Cooperation and Development, OECD Guidelines for Testing of Chemicals Guideline 425 Acute Oral Toxicity–Up-and-Down Method Approved June 1998

(4) U.S Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision F Health Effects EPA report 540/09–82–025, October 1982

(5) U S Environmental Protection Agency Pesticide Reregistration Rejection Rate Analysis Toxicology EPA report 738–R–93–004 July 1993

(6) U S Environmental Protection Agency *Conduct of Acute Toxicity Studies* EPA report 737–R–97–002 September 1997

(7) U S Environmental Protection Agency *Label Review Manual* 2nd Edition EPA report 737–B–96–001 December 1996

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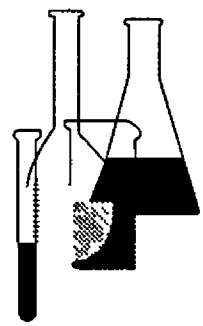
Health Effects Test Guidelines OPPTS 870.1100 Acute Oral Toxicity



INTRODUCTION

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OPPTS 870 1100 Acute oral toxicity

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 798 1175 Acute Oral Toxicity, OPP 81–1 (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation; Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 401 Acute Oral Toxicity

(b) **Purpose.** In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step It provides information on health hazards likely to arise from short-term exposure by the oral route Data from an acute study may serve as a basis for classification and labeling It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance An evaluation of acute toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects

(c) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline The following definitions also apply to this test guideline

Acute oral toxicity is the adverse effects occurring within a short period of time after oral administration of either a single dose of a substance or multiple doses given within a 24–hour period

Dosage is a general term comprising the dose, its frequency, and the duration of dosing

Dose is the amount of test substance administered. Dose is expressed as weight of test substance (milligrams, grams) per unit weight of test animal (e g. milligrams per kilogram)

Dose-effect is the relationship between the dose and the magnitude of a defined biological effect either in an individual or in a population sample

Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect

LD_{50} (median lethal dose) is a statistically derived estimate of single dose of a substance that can be expected to cause death in 50 percent

of animals when administered by the oral route The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram)

(d) **Approaches to the determination of acute toxicity.** (1) EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety

(i) Use of appropriate alternative test protocols when available Thus, for example, acute oral toxicity testing may be performed using the Fixed Dose Method (OECD Guideline 420, see paragraph (f)(1) of this guideline), or the Acute Toxic Class Method (OECD Guideline 423, see aragraph (f)(2) of this guideline), or the Up-and-Down Method (OECD Guideline 425, see paragraph (f)(3) of this guideline) Abbreviated methods are not yet available through OECD for acute toxicity by other routes of exposure However, OECD is actively revising its approaches to testing for dermal and ocular irritation and when this is done, the OPPTS guidelines will be updated to harmonize with the OECD revisions

(ii) Limit test. When data on structurally related chemicals are inadequate, a limit test may be considered If rodents are used, a limit dose of at least 2,000 mg per kilogram of body weight may be administered to a single group of five males and five females using the procedures described under paragraph (e) of this guideline If no lethality is demonstrated, no further testing for acute oral toxicity is needed. (Under current policy and regulations for pesticide products, precautionary statements may still be required unless there are data to indicate the LD_{50} is greater than 5,000 mg/kg) If compound-related mortality is produced in the limit test, further study may need to be considered.

(iii) Estimation of acute oral toxicity When further study is warranted, EPA generally supports limiting such tests to those using the lowest number of animals feasible Given the approval internationally through OECD of three alternative test methods to the ''traditional'' acute oral toxicity test, it is time to reassess the status of acute toxicity testing The three OECD alternatives include the following The fixed dose procedure is a refinement of the traditional acute oral test that employs nonlethal endpoints. In contrast, the acute toxic class and up-and-down procedures estimate lethality within a dose range and as a point estimate, respectively, and reduce animal usage in comparison to the ''traditional'' test.

(A) The up and down procedure as described in OECD Guideline 425 referenced in paragraph (f)(4) of this guideline This method is highly recommended

(B) The acute toxic class method as described in OECD Guideline 423 and referenced in paragraph (f)(6) of this guideline

(C) A three-dose method described as the conventional acute toxicity test under paragraph (e) in this guideline, and in OECD Guideline 401 referenced in paragraph (f)(7) of this guideline

(D) The fixed dose method as described in OECD Guideline 420 and referenced in paragraph (f)(5) of this guideline

(e) **Conventional acute toxicity test**—(1) **Principle of the test method.** The test substance is administered orally by gavage in graduated doses to several groups of experimental animals, one dose being used per group The doses chosen may be based on the results of a range finding test Subsequently, observations of effects and deaths are made Animals that die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied This guideline is directed primarily to studies in rodent species but may be adapted for studies in nonrodents Animals showing severe and enduring signs of distress and pain may need to be humanely killed Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out

(2) **Substance to be tested.** Test, control and reference substances are discussed in 40 CFR Part 792—Good Laboratory Practice Standards.

(3) **Test procedures**—(i) **Preparations.** Healthy young adult animals are acclimatized to the laboratory conditions for at least 5 days prior to the test before the test animals are randomized and assigned to the treatment groups

(ii) **Animal selection**—(A) **Species and strain.** Although several mammalian test species may be used, the rat is the preferred species Commonly used laboratory strains should be employed If another species is used, the tester should provide justification and reasoning for its selection.

(B) **Age.** Young adult rats between 8– and 12–weeks–old at the beginning of dosing should be used Rabbits should be at least 12 weeks of age at study initiation The weight variation of animals used in a test should be within 20 percent of the mean weight for each sex

(C) **Number and sex of animals.** (*1*) At least five experimentally naive rodents are used at each dose level They should all be of the same sex After completion of the study in one sex, at least one group of five animals of the other sex is dosed to establish that animals of this sex are not markedly more sensitive to the test substance The use of fewer animals may be justified in individual circumstances Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex may be dispensed with An acceptable option would be to test at least one group of five animals per sex at one or more dose levels to definitively determine the more sensitive sex prior to conducting the main study

(*2*) The females should be nulliparous and nonpregnant

(*3*) In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered

(D) **Assignment of animals.** Each animal must be assigned a unique identification number A system to assign animals to test groups and control groups randomly is required

(E) **Housing.** Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e g morbidity, excitability) may indicate a need for individual caging

(*1*) The temperature of the experimental animal rooms should be at 22 ± 3 °C for rodents

(*2*) The relative humidity of the experimental animal rooms should be 30 to 70 percent

(*3*) Where lighting is artificial, the sequence should be 12–hours light/ 12–hours dark.

(*4*) For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(iii) **Dose levels and dose selection.** (A) Three dose levels should be used, spaced appropriately to produce test groups with a range of toxic effects and mortality rates The data should be sufficient to produce a dose-response curve and permit an acceptable estimation of the LD_{50} Range finding studies using single animals may help to estimate the positioning of dose groups so that no more than three dose levels will be necessary. An acceptable option for pesticide products would be to set the dose levels in correlation with the OPP toxicity categories (bracketing) In these cases, the determination of an LD_{50} may not be necessary

(B) Limit test. This test has been defined and described under paragraph (d)(2)(ii) of this guideline

(C) Vehicle. Where necessary, the test substance is dissolved or suspended in a suitable vehicle If a vehicle or diluent is needed, it should not elicit toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of a solution in oil (e g , corn oil), and then by consideration of possible solution in other vehicles Toxic characteristics of nonaqueous vehicles should be known, and, if not known, should be determined before the test

(D) Volume The maximum volume of liquid that can be administered at one time depends on the size of the test animal In rodents, the volume should not exceed 1 mL/100 g body weight, except when an aqueous solution is used in which case 2 mL/100 g may be administered Either constant volume or constant concentration administration is acceptable when dosing, provided the following guidance is employed When possible, the liquid test material should be dosed neat Otherwise, it may be diluted, using the highest concentration possible, although volumes less than 0 5 mL per animal would not be required Lower dose volumes are acceptable if they can be accurately administered Solid materials should be suspended or dissolved in the minimum amount of vehicle and dosed at the highest concentration possible

(iv) **Exposure and exposure duration.** (A) Animals should be fasted prior to test substance administration For the rat, feed should be withheld overnight, for other rodents with higher metabolic rates a shorter period of fasting is appropriate

(B) The test substance should be administered in a single dose by gavage, using a stomach tube or suitable intubation cannula

(C) If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours Where a dose is administered in fractions, it may be necessary to provide the animals with food and water, depending on the length of the dosing period

(D) After the substance has been administered, feed may be withheld for an additional 3–4 hours

(v) **Observation period.** Although 14 days is recommended as a minimum observation period, the duration of observation should not be fixed rigidly It should be determined by the toxic reactions, rate of onset, and length of recovery period, and may thus be extended when considered necessary The time at which signs of toxicity appear, their duration, and the time to death are important, especially if there is a tendency for deaths to be delayed

(vi) **Observation of animals.** (A) A careful clinical examination should be made at least once each day

(B) Additional observations should be made daily, especially in the early days of the study Appropriate actions should be taken to minimize loss of animals to the study (e g , necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals)

(C) Observations should be detailed and carefully recorded, preferably using explicitly defined scales Observations should include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation,

central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli altered strength, and stereotypies or bizarre behavior (e g , self-mutilation, walking backwards)

(D) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death Changes in weights should be calculated and recorded when survival exceeds 1 day

(E) The time of death should be recorded as precisely as possible

(vii) **Gross pathology.** (A) At the end of the test, surviving animals should be weighed and sacrificed

(B) A gross necropsy should be performed on all animals under test All gross pathology changes should be recorded

(C) If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at temperatures low enough to minimize autolysis Necropsies should be performed as soon as practicable, normally within a day or two.

(viii) **Additional evaluation.** Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 hours or more should also be considered because it may yield useful information

(ix) **Data and reporting**—(A) **Treatment of results.** Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects, and necropsy findings Any methods used for calculation of the LD_{50} or any other parameters should be specified and referenced Methods for parameter estimation are described under paragraphs (f)(1), (f)(2), and (f)(3) of this guideline

(B) **Evaluation of results.** An evaluation should include the relationship, if any, between exposure of the animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects The LD_{50} value should always be considered in conjunction with the observed toxic effects and any necropsy findings The LD_{50} value is a relatively coarse measurement, useful only as a reference value for classification and labeling purposes, and for an expression of the lethal potential of the test substance by the ingestion route Reference should always be made to the experimental animal species in which the LD_{50} value was obtained

(C) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J and 40 CFR part 160, subpart J, the

following specific information should be reported The test report should include

(*1*) Species, strain, sex, and source of test animals

(*2*) Method of randomization in assigning animals to test and control groups

(*3*) Rationale for selection of species, if other than that recommended

(*4*) Tabulation of individual and test group data by sex and dose level (e g number of animals exposed, number of animals showing signs of toxicity and number of animals that died or were killed during the test)

(*i*) Description of toxic effects, including their time of onset, duration, reversibility, and relationship to dose

(*ii*) Body weights

(*iii*) Time of dosing and time of death after dosing

(*iv*) Dose-response curves for mortality and other toxic effects (when permitted by the method of determination)

(*v*) Gross pathology findings

(*vi*) Histopathology findings and any additional clinical chemistry evaluations, if performed

(*5*) Description of any pretest conditioning, including diet, quarantine and treatment for disease

(*6*) Description of caging conditions including Number (or change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animals

(*7*) Manufacturer, source, purity, and lot number of test substance

(*8*) Relevant properties of substance tested including physical state and pH (if applicable)

(*9*) Identification and composition of any vehicles (e.g , diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance

(*10*) A list of references cited in the body of the report References to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results

(f) **References.** The following references should be consulted for additional background material on this test guideline

(1) Chanter, D O and Heywood, R The LD_{50} Test Some Considerations of Precision *Toxicology Letters* 10 303–307 (1982)

(2) Finney, D J Chapter 3—Estimation of the median effective dose and Chapter 4—Maximum likelihood estimation, *Probit Analysis*, 3rd ed Cambridge, London (1971)

(3) Finney, D J The Median Lethal Dose and Its Estimation. *Archives of Toxicology* 56 215–218 (1985)

(4) Organization for Economic Cooperation and Development. OECD Guidelines for the Testing of Chemicals OECD Guideline 425 Acute Oral Toxicity Up-and-Down Procedure, Approved June 1998

(5) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 420. Acute Oral Toxicity—Fixed Dose Method, Adopted July 17, 1992

(6) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 423 Acute Oral Toxicity - Acute Toxic Class Method, Adopted March 22, 1996

(7) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 401 Acute Oral Toxicity, Adopted February 24, 1987

United States
Environmental Protection
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Prevention Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–192
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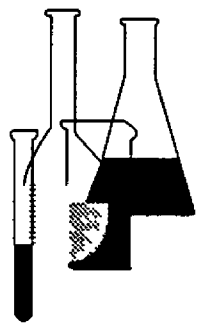
Health Effects Test Guidelines OPPTS 870.1200 Acute Dermal Toxicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S. Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.1200 Acute dermal toxicity.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 798 1100 Acute Dermal Toxicity, OPP 81–2 Acute Dermal Toxicity (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 402 Acute Dermal Toxicity

(b) **Purpose.** In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely It provides information on health hazards likely to arise from short-term exposure by the dermal route Data from an acute study may serve as a basis for classification and labeling It is an initial step in establishing a dosage regimen in subchronic and other studies and may provide information on dermal absorption and the mode of toxic action of a substance by this route An evaluation of acute toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects

(c) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline

Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a substance or multiple doses given within a 24–h period

Dosage is a general term comprising the dose, its frequency and the duration of dosing

Dose is the amount of test substance applied Dose is expressed as weight of test substance (grams, milligrams) per unit weight of test animal (e g milligrams per kilogram)

Dose-effect is the relationship between the dose and the magnitude of a defined biological effect either in an individual or in a population sample

Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect

LD_{50} (median lethal dose), dermal, is a statistically derived estimate of a single dose of a substance that can be expected to cause death in 50 percent of treated animals when applied to the skin The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram)

(d) **Approaches to the determination of acute toxicity.** (1) EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety

(i) Using data from substantially similar mixtures In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on mixtures that are substantially similar to the mixture under investigation In certain cases it may be possible to glean enough information to make preliminary hazard evaluations that may reduce the need for further animal testing

(ii) Limit test When data on structurally related chemicals are inadequate, a limit test may be considered If rodents are used, a limit dose of at least 2,000 mg/kg bodyweight may be administered to a single group of five males and five females using the procedures described under paragraph (e) of this guideline If no lethality is demonstrated, no further testing for acute dermal toxicity is needed (Under current policy for pesticide products, precautionary statements may still be required unless there are data to indicate the LD_{50} is greater than 5,000 mg/kg) If compound-related mortality is produced, further study may need to be considered.

(2) [Reserved]

(e) **Conventional acute toxicity test—(1) Principle of the test method.** The test substance is applied dermally in graduated doses to several groups of experimental animals, one dose being used per group. The doses chosen may be based on the results of a range finding test. Subsequently, observations of effects and deaths are made Animals that die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied This guideline is directed primarily to studies in either rats, rabbits, or guinea pigs but may be adapted for studies in other species Animals showing severe and enduring signs of distress and pain may need to be humanely killed Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out

(2) **Substance to be tested.** Test, control, and reference substances are discussed in 40 CFR Part 792—Good Laboratory Practice Standards

(3) **Test procedures—(i) Preparations.** Healthy young adult animals are acclimatized to the laboratory conditions for at least 5 days prior to

the test before the test animals are randomized and assigned to the treatment groups

(ii) **Animal selection**—(A) **Species and strain.** The rat, rabbit, or guinea pig may be used The albino rabbit is preferred because of its size, ease of handling, skin permeability, and extensive data base Commonly used laboratory strains should be employed If a species other than rats, rabbits, or guinea pigs is used, the tester should provide justification and reasoning for its selection

(B) **Age** Young adult animals, rats between 8– and 12–weeks–old, rabbits at least 12–weeks–old, and guinea pigs between 5– and 6–weeks–old at the beginning of dosing should be used The weight variation of animals used in a test should be within 20 percent of the mean weight for each sex

(C) **Number and sex of animals.** (*1*) At least five experimentally naive animals with healthy intact skin are used at each dose level They should all be of the same sex After completion of the study in one sex, at least one group of five animals of the other sex is dosed to establish that animals of this sex are not markedly more sensitive to the test substance. The use of fewer animals may be justified in individual circumstances Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex may be dispensed with An acceptable option would be to test at least one group of five animals per sex at one or more dose levels to definitively determine the more sensitive sex prior to conducting the main study

(*2*) The females should be nulliparous and nonpregnant

(*3*) In acute toxicity tests with animals of a higher order than those mentioned above, the use of smaller numbers should be considered

(D) **Assignment of animals.** Each animal must be assigned a unique identification number A system to randomly assign animals to test groups and control groups is required

(E) **Housing.** Animals should be housed in individual cages

(*1*) The temperature of the experimental animal rooms should be at 22±3 °C for rodents, 20±3 °C for rabbits

(*2*) The relative humidity of the experimental animal rooms should be 30 to 70 percent.

(*3*) Where lighting is artificial, the sequence should be 12–h light/ 12–h dark

(*4*) For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(iii) **Dose levels and dose selection.** (A) Three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates The data should be sufficient to produce a dose-response curve and permit an acceptable estimation of the median lethal dose Range finding studies using single animals may help to estimate the positioning of the dose groups so that no more than three dose levels will be necessary An acceptable option for pesticide products would be to set the dose levels in correlation with the OPP toxicity categories (bracketing). In these cases, the determination of an LD_{50} may not be necessary

(B) Limit test This test is described under paragraph (d)(2)(ii) of this guideline

(C) Vehicle Solids should be pulverized when possible The test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with skin If a vehicle or diluent is needed, it should not elicit toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. In addition, the influence of the vehicle on penetration of skin by the test substance should be taken into account It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of a solution in oil (e g corn oil), and then by consideration of possible solution in other vehicles For nonaqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test Acceptable alternative vehicles include gum arabic, ethanol and water, carboxymethyl cellulose, glycerol, propylene glycol, PEG vegetable oil, and mineral oil as long as the vehicle is not irritating and the inability to use water or saline is justified in the report

(iv) **Exposure and exposure duration.** The test substance should be administered over a period of 24 h

(v) **Preparation of animal skin.** Fur should be clipped from the dorsal area of the trunk of the test animals Shaving may be employed, but it should be carried out at least 24 h before dosing Care must be taken to avoid abrading the skin, which would alter its permeability

(vi) **Application of test substance.** (A) The test substance should be applied uniformly over a shaved or clipped area which is approximately 10 percent of the body surface area The area starting at the scapulae (shoulders) to the wing of the ileum (hip bone) and half way down the flank on each side of the animal should be shaved or clipped. Liquid test materials should be undiluted if possible With highly toxic substances, the surface area covered may be less, but as much of the area as possible should be covered with as thin and uniform a film as practical The test

material is not removed until 24 h after application In the case where less than 10 percent of the surface area is covered an approximation of the exposed areas should be determined

(B) The test substance should be held in contact with the skin with a porous gauze dressing (<8 ply) and nonirritating tape throughout a 24–h exposure period The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method Although a semiocclusive dressing is preferred, an occlusive dressing will also be acceptable

(C) At the end of the exposure period, residual test substance should be removed where practicable using water or an appropriate solvent

(vii) **Observation period.** Although 14 days is recommended as a minimum observation period, the duration of observation should not be fixed rigidly It should be determined by the toxic reactions, rate of onset, and length of recovery period and may thus be extended when considered necessary The time at which signs of toxicity appear, their duration, and the time to death are important, especially if there is a tendency for deaths to be delayed

(viii) **Observation of animals.** (A) A careful clinical examination should be made at least once each day

(B) Additional observations should be made daily, especially in the early days of the study Appropriate actions should be taken to minimize loss of animals to the study (e g necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

(C) Observations should be detailed and carefully recorded, preferably using explicitly defined scales Observations should include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypies or bizarre behavior (e g self-mutilation, walking backwards)

(D) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death Changes in weights should be calculated and recorded when survival exceeds one day

(E) The time of death should be recorded as precisely as possible

(ix) **Gross pathology.** (A) At the end of the test, surviving animals should be weighed and sacrificed

(B) A gross necropsy should be performed on all animals under test All gross pathology changes should be recorded

(C) If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at temperatures low enough to minimize autolysis Necropsies should be performed as soon as practicable, normally within a day or two

(x) **Additional evaluations.** Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 h or more should also be considered because it may yield useful information

(xi) **Data and reporting**—(A) **Treatment of results.** Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings. Any methods used for calculation of the LD_{50} or any other parameters should be specified and referenced Methods for parameter estimation are described under paragraphs (f)(1), (f)(2), and (f)(3) of this guideline

(B) **Evaluation of results.** An evaluation should include the relationship, if any, between exposure of the animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects The LD_{50} value should always be considered in conjunction with the observed toxic effects and any necropsy findings. The LD_{50} value is a relatively coarse measurement, useful only as a reference value for classification and labeling purposes, and for an expression of the lethal potential of the test substance by the ingestion route Reference should always be made to the experimental animal species in which the LD_{50} value was obtained

(C) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J and 40 CFR part 160, subpart J, the following specific information should be reported The test report should include

(*1*) Species, strain, sex, and source of test animals

(*2*) Method of randomization in assigning animals to test and control groups

(*3*) Rationale for selection of species, if other than that recommended

(*4*) Tabulation of individual and test group data by sex and dose level (e g number of animals exposed, number of animals showing signs of toxicity and number of animals that died or were killed during the test)

(*i*) Description of toxic effects, including their time of onset, duration, reversibility, and relationship to dose

(*ii*) Body weights

(*iii*) Time of dosing and time of death after dosing

(*iv*) Dose-response curves for mortality and other toxic effects (when permitted by the method of determination)

(*v*) Gross pathology findings

(*vi*) Histopathology findings and any additional clinical chemistry evaluations, if performed

(*5*) Description of any pre-test conditioning, including diet, quarantine and treatment for disease

(*6*) Description of caging conditions including Number (or change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animals

(*7*) Manufacturer, source, purity, and lot number of test substance

(*8*) Relevant properties of substance tested including physical state and pH (if applicable)

(*9*) Identification and composition of any vehicles (e g , diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance

(*10*) A list of references cited in the body of the report. References to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.

(f) **References.** The following references should be consulted for additional background information on this test guideline

(1) Chanter, D O and Heywood, R , The LD_{50} Test Some Considerations of Precision, *Toxicology Letters* 10 303–307 (1982)

(2) Finney, D J Chapter 3—Estimation of the median effective dose and Chapter 4-Maximum likelihood estimation, *Probit Analysis*, 3rd ed Cambridge, London (1971)

(3) Finney, D J The Median Lethal Dose and Its Estimation *Archives of Toxicology* 56 215–218 (1985)

(4) Organization for Economic Cooperation and Development OECD Guidelines for the Testing of Chemicals Final Draft OECD Guideline 425

Acute Oral Toxicity Up-and-Down Procedure to be adopted in the Tenth Addendum to the OECD Guidelines for the Testing of Chemicals

(5) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 420 Acute Oral Toxicity—Fixed Dose Method Adopted July 17, 1992

(6) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 423 Acute Oral Toxicity—Acute Toxic Class Method Adopted March 22, 1996

(7) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 402 Acute Dermal Toxicity Adopted· February 24, 1987

United States
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Agency

Prevention Pesticides
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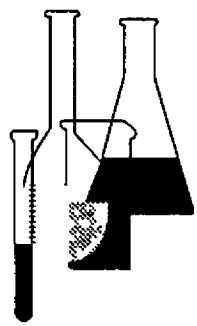
Health Effects Test Guidelines OPPTS 870.1300 Acute Inhalation Toxicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S. Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.1300 Acute inhalation toxicity

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 798 1150 Acute Inhalation Toxicity, OPP 81–3 Acute Inhalation Toxicity-Rat(Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD guideline 403 Acute Inhalation Toxicity

(b) **Purpose.** Determination of acute toxicity is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance that may be inhaled such as a gas, volatile substance, or aerosol/particle It provides information on health hazards likely to arise from short-term exposure by the inhalation route Data from an acute study may serve as a basis for classification and labeling It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects

(c) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline The following definitions also apply to this test guideline

Acute inhalation toxicity is the adverse effect caused by a substance following a single uninterrupted exposure by inhalation over a short period of time (24 h or less) to a substance capable of being inhaled

Aerodynamic equivalent diameter is defined as the diameter of a unit-density sphere having the same terminal settling velocity as the particle in question, whatever its size, shape, and density It is used to predict where in the respiratory tract such particles may be deposited

Concentration is expressed as weight of the test substance per unit volume of air, e g milligrams per liter

Inhalable diameter refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism under study It is used to refer to particles which are capable of being inhaled and deposited anywhere within the respiratory tract

LC_{50} (median lethal concentration) is a statistically derived estimate of a concentration of a substance that can be expected to cause death during exposure or within a fixed time after exposure in 50 percent of animals exposed for a specified time The LC_{50} value is expressed as weight of test substance per unit volume of air (milligrams per liter) or parts per million For clarity, the exposure duration should also be specified, e g 4–h LC_{50}

Mass median aerodynamic diameter (MMAD) is the median aerodynamic diameter and, along with the geometric standard deviation, is used to describe the particle size distribution of any aerosol statistically, based on the weight and size of the particles Fifty percent of the particles by weight will be smaller than the median diameter and 50 percent of the particles will be larger

(d) **Approaches to the determination of acute toxicity.** (1) EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety

(i) Using data from substantially similar mixtures In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on mixtures that are substantially similar to mixtures under investigation In certain cases, it may be possible to get enough information to make preliminary hazard evaluations that may reduce the need for further animal testing

(ii) Limit test When data on structurally related chemicals are inadequate, a limit test may be considered In the limit test, a single group of five males and five females is exposed to 2 mg/L for 4 h, or where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, using the procedures described under paragraph (e) of this guideline If no lethality is demonstrated, no further testing for acute inhalation toxicity is needed If compound-related mortality is produced, further study may need to be considered

(2) [Reserved]

(e) **Conventional acute toxicity test—(1) Principle of the test method.** Several groups of experimental animals are exposed to the test substance in graduated concentrations for a defined period, one concentration being used per group When a vehicle other than water is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group should be used when historical data are not available or adequate to determine the acute inhalation toxicity of the vehicle Subsequently, observations of effects and death are made Animals that die during the test are necropsied and at the conclusion of the test surviving animals are sacrificed and necropsied This guideline is directed pri-

marily to studies in rodent species but may be adapted for studies in non-rodents Animals showing severe and enduring signs of distress and pain may need to be humanely killed Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out

(2) **Substance to be tested.** Test, control, and reference substances are discussed in 40 CFR part 792, subpart F (Good Laboratory Practice Standards)

(3) **Test procedure**—(i) **Preparation.** Healthy young adult animals are acclimatized to the laboratory conditions for at least 5 days prior to the test Before the test, animals are randomized and assigned to the required number of groups

(ii) **Animal selection**—(A) **Species and strain.** Although several mammalian test species may be used, the preferred species is the rat Commonly used laboratory strains should be employed If another mammalian species is used, the tester should provide justification and reasoning for its selection

(B) **Age.** Young adult rats between 8–12 weeks old at the beginning of dosing, should be used The weight variation in animals or between groups used in a test should not exceed ±20 percent of the mean weight of each sex

(C) **Number and sex.** (*1*) At least five experimentally naive animals are used at each concentration and they should be of one sex After completion of the study in one sex, at least one group of five animals of the other sex is exposed to establish that animals of this sex are not markedly more sensitive to the test substance The use of fewer animals may be justified in individual circumstances Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex is not required An acceptable option would be to test at least one group of five animals per sex at one or more dose levels to definitively determine the more sensitive sex prior to conducting the main study

(2) Females should be nulliparous and nonpregnant.

(*3*) In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered

(D) **Assignment of animals.** Each animal must be assigned a unique identification number A system to assign animals to test groups and control groups randomly is required

(E) **Housing.** The animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal The biological properties of the test substance or toxic effects (e g

morbidity, excitability) may indicate a need for individual caging Animals must be housed individually in inhalation chambers during exposure to aerosols

(*1*) Before and after exposure, the temperature of the animal room should be 22 ± 3 °C and the relative humidity 30–70 percent

(*2*) Where lighting is artificial, the sequence should be 12 h light/ 12 h dark.

(*3*) For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(F) **Equipment.** (*1*) The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of at least 10 air changes per hour, an adequate oxygen content of at least 19 percent, and uniform conditions throughout the exposure chamber Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas It is normally not necessary to measure chamber oxygen concentration if airflow is adequate

(2) The selection of a dynamic inhalation chamber should be appropriate for the test article and test system Where a whole body chamber is used to expose animals to an aerosol, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance To ensure stability of a chamber atmosphere, the total volume of the test animals should not exceed 5 percent of the volume of the test chamber It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order to minimize oral exposures due to animals licking compound off their fur The animals should be acclimated and heat stress minimized

(G) **Physical measurements.** Measurements or monitoring should be made of the following:

(*1*) The rate of air flow should be monitored continuously, but recorded at least 3 times during the exposure

(2) The actual concentrations of the test substance should be measured in the breathing zone During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable and monitored continuously or intermittently depending on the method of analysis Chamber concentration may be measured using gravimetric or analytical methods as appropriate. If trial run measurements are reasonably consistent (±10 percent for liquid aerosol, gas, or vapor, ±20 percent for dry aerosol), then two measurements should be sufficient If measurements are not consistent, three to four measurements should be taken Whenever the test article is a formulation, the analytical concentration must be reported for the total formulation, and not just for the active ingredient (AI) If,

for example, a formulation contains 10 percent AI and 90 percent inerts, a chamber analytical limit concentration of 2 mg/L would consist of 0 2 mg/L of the AI It is not necessary to analyze inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation, the grounds for this conclusion must be provided in the study report If there is some difficulty in measuring chamber analytical concentration due to precipitation, nonhomogeneous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary

(*3*) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations The MMAD particle size range should be between 1–4 μm The particle size of hygroscopic materials should be small enough when dry to assure that the size of the swollen particle will still be within the 1–4 μm range Measurements of aerodynamic particle size in the animal's breathing zone should be measured during a trial run If MMAD values for each exposure level are within 10 percent of each other, then two measurements during the exposures should be sufficient If pretest measurements are not within 10 percent of each other, three to four measurements should be taken

(*4*) Temperature and humidity should be monitored continuously and recorded at least 3 times during exposure The temperature at which the test is performed should be maintained at 22±2 °C The relative humidity should be maintained between 30 and 70 percent humidity, but in certain instances (tests of aerosols) this may not be practicable

(iii) **Exposure duration and levels.** (A) Shortly before exposure, the animals are weighed and then exposed to the test concentration in the designated apparatus for 4 h after equilibration of the chamber concentrations Other durations may be needed to meet specific requirements. Food should be withheld during exposure Water may also be withheld in certain circumstances

(B) **Exposure levels.** Three concentration levels should be used and spaced appropriately to produce a concentration-response curve and permit an estimation of the median lethal concentration Range-finding studies using single animals may help to estimate the positioning of the test groups so that no more than three concentration levels will be necessary An acceptable option for pesticide products would be to set the dose levels in correlation with the OPP toxicity categories (bracketing) In these cases, the determination of an LD_{50} may not be necessary

(iv) **Observation period.** The observation period should be at least 14 days However, the duration of observation should not be fixed rigidly It should be determined by the toxic reactions, rate of onset, and length of recovery period, and thus may be extended when considered necessary The time at which signs of toxicity appear, their duration, and the time

of death are important, especially if there is a tendency for deaths to be delayed

(v) **Observation of animals** (A) A careful clinical examination should be made at least once each day

(B) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e g. necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals

(C) Observations should be detailed and carefully recorded, preferably using explicitly defined scales Observations should include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypies or bizarre behavior (e g , self mutilation, walking backwards)

(D) Individual weights of animals should be determined prior to exposure, weekly after exposure, and at death Changes in weights should be calculated and recorded when survival exceeds 1 day

(E) The time of death should be recorded as precisely as possible

(vi) **Gross pathology.** (A) At the end of the test, surviving animals should be weighed and sacrificed

(B) A gross necropsy should be performed on all animals under test, with particular reference to any changes in the respiratory tract. All gross pathology changes should be recorded

(C) If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at temperatures low enough to minimize autolysis Necropsies should be performed as soon as possible, normally within a day or two

(viii) **Additional evaluations.** In animals surviving 24 h or more, microscopic examination of organs showing evidence of gross pathology should be considered since it may yield useful information.

(ix) **Data and reporting**—(A) **Treatment of results.** Data should be summarized in tabular form showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different exposure levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings Any method used for calculation of the LC_{50} or any other parameters should be specified and referenced Methods for parameter estimation are described under paragraphs (f)(1), (f)(2), and (f)(3) of this guideline

(B) **Evaluation of results.** The LC_{50} value should be considered in conjunction with the observed toxic effects and the necropsy findings The LC_{50} value is a relatively coarse measurement useful only for classification and labeling purposes and an expression of the lethal potential of the test substance following inhalation Reference should always be made to the experimental animal species and exposure duration in which the LC_{50} value was obtained An evaluation should include the relationship, if any, between exposure of animals to the test substance and the incidence and severity of all abnormalities including behavioral and clinical abnormalities, gross lesions, body weight changes, mortality, and other toxic effects

(C) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J and 40 CFR 160, subpart J, the following specific information should be reported

(*1*) Test conditions (*i*) Description of exposure apparatus including design, type, dimensions

(*ii*) Source of air, system for generating the test article

(*iii*) Equipment for measuring temperature, humidity, particle size, and actual concentration

(*iv*) Treatment of exhaust air and the method of housing the animals in a test chamber when this is used

(*2*) Exposure data These should be tabulated and presented with mean values and a measure of variability (e g standard deviation) and should include

(*i*) Airflow rates through the inhalation equipment

(*ii*) Temperature and humidity of the air

(*iii*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air)

(*iv*) Actual (analytical or gravimetric) concentration in test breathing zone

(*v*) Particle size distribution, and calculated MMAD and geometric standard deviation (GSD)

(*vi*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable), and the efforts taken to comply with these aspects of the guidelines

(*3*) Species, strain, sex, and source of test animals

(*4*) Method of randomization in assigning animals to test and control groups

(5) Rationale for selection of species, if other than that recommended

(6) Tabulation of individual and test group data by sex and exposure level (e g number of animals exposed, number of animals showing signs of toxicity and number of animals that died or were killed during the test)

(i) Description of toxic effects including their time of onset, duration, reversibility, and relationship to concentration

(ii) Body weights

(iii) Time of dosing and time of death during or following exposure

(iv) Concentration-response curves for mortality and other toxic effects (when permitted by the method of determination)

(v) Gross pathology findings

(vi) Histopathology findings and any additional clinical chemistry evaluations if performed

(7) Description of any pretest conditioning, including diet, quarantine and treatment for disease

(8) Description of caging conditions, including number (or change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animals

(9) Manufacturer (source), lot number, and purity of test substance

(10) Identification and composition of any vehicles (e.g , diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance

(11) A list of references cited in the body of the report. References to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results

(f) **References.** The following references should be consulted for additional background material on this test guideline

(1) Chanter, D.O. and Heywood, R The LD_{50} test: some considerations of precision *Toxicology Letters* 10·303–307 (1982)

(2) Finney, D G Chapter 3—Estimation of the median effective dose, Chapter 4—Maximum likelihood estimation *Probit Analysis* 3rd Ed (Cambridge, London (1971)

(3) Finney, D J The Median Lethal Dose and Its Estimation, *Archives of Toxicology* 56 215–218 (1985)

(4) Organization for Economic Cooperation and Development OECD Guidelines for the Testing of Chemicals Final Draft OECD Guideline 425 Acute Oral Toxicity Up-and-Down Procedure to be adopted in the Tenth Addendum to the OECD Guidelines for the Testing of Chemicals

(5) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 403 Acute Inhalation Toxicity. Adopted May 12, 1981

(6) Organization for Economic Cooperation and Development. OECD Guidelines for Testing of Chemicals Guideline 420 Acute Oral Toxicity—Fixed Dose Method Adopted July 17, 1992

(7) Organization for Economic Cooperation and Development OECD Guidelines for Testing of Chemicals Guideline 423 Acute Oral Toxicity—Acute Toxic Class Method Adopted March 22, 1996

(8) U S EPA Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies 2/1/94 Health Effects Division, Office of Pesticide Programs

United States
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Prevention Pesticides
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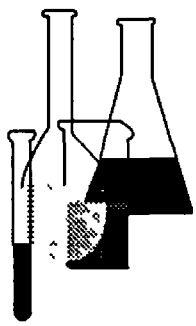
Health Effects Test Guidelines OPPTS 870.2400 Acute Eye Irritation



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U.S C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S C 136, *et seq*)

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OPPTS 870 2400 Acute eye irritation.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPTS 798 4500 Primary Eye Irritation, OPP 81–4 Acute Eye Irritation—Rabbit (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 405 Acute Eye Irritation/ Corrosion

(b) **Purpose.** (1) In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on eyes of mammals is an important initial step Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance

(2) Data on primary eye irritation are required by 40 CFR 158 340 to support the registration of each manufacturing-use product and end-use product (See § 158 50 to determine whether these data must be submitted and which purity/grade of the test substance should be tested)

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

Eye corrosion is the production of irreversible tissue damage in the eye following application of a test substance to the anterior surface of the eye

Eye irritation is the production of reversible changes in the eye following the application of a test substance to the anterior surface of the eye.

(d) **Principle of the test method.** The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals, the untreated eye is used to provide control information The degree of irritation/corrosion is evaluated and scored at specified intervals and is fully described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed The period of observation should be at least 72 h, but need not exceed 21 days Animals showing severe and enduring signs of distress and pain may need to be killed in a humane fashion

(e) **Initial considerations.** (1) Strongly acidic or alkaline substances, for example, with a demonstrated pH of 2 or less or 11 5 or greater, need not be tested owing to their predictable corrosive properties Buffer capacity should also be taken into account

(2) Materials which have demonstrated definite corrosion or severe irritation in a dermal study need not be further tested for eye irritation It may be presumed that such substances will produce similarly severe effects in the eyes.

(3) Results from well validated and accepted *in vitro* test systems may serve to identify corrosives or irritants such that the test material need not be tested *in vivo*

(f) **Test procedures—(1) Animal selection—(i) Species and strain.** A variety of experimental animals has been used, but it is recommended that testing should be performed using healthy adult albino rabbits. Commonly used laboratory strains should be used If another mammalian species is used, the tester should provide justification/reasoning for its selection.

(ii) **Number of animals.** A single animal should be considered if marked effects are anticipated If the results of this test in one animal suggest the test substance to be a severe irritant (reversible effect) or corrosive (irreversible effect) to the eye using the procedure described, further tests may not need to be performed In cases other than a single animal test, at least three animals should be used Occasionally, further testing in additional animals may be appropriate to clarify equivocal responses

(2) **Dose level.** For testing liquids, a dose of 0 1 mL is recommended In testing solids, pastes, and particulate substances, the amount used should have a volume of 0 1 mL, or a weight of not more than 100 mg (the weight must always be recorded). If the test material is solid or granular, it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them (e g by tapping the measuring container). To test a substance contained in a pressurized aerosol container, the eye should be held open and the test substance administered in a single burst of about 1 sec from a distance of 10 cm directly in front of the eye The dose may be estimated by weighing the container before and after use Care should be taken not to damage the eye. Pump sprays should not be used but instead the liquid should be expelled and 0 1 mL collected and instilled into the eye as described for liquids For volatile substances, the dose may be estimated by weighing the container before and after use.

(3) **Examination of eyes prior to test.** Both eyes of each experimental animal provisionally selected for testing should be examined within 24 h before testing starts by the same procedure to be used during the

test examination Animals showing eye irritation, ocular defects, or pre-existing corneal injury should not be used

(4) **Application of the test substance** (i) The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball The lids are then gently held together for about 1 sec in order to limit loss of the material The other eye, which remains untreated, serves as a control If it is thought that the substance may cause extreme pain, local anesthetic may be used prior to instillation of the test substance The type and concentration of the local anesthetic, should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anesthetized

(ii) The eyes of the test animals should not be washed out for 24 h following instillation of the test substance At 24 h, a washout may be used if considered appropriate This is to show whether washing with water palliates or exacerbates irritation

(iii) For some substances shown to be irritating by this test, additional testing using animals with eyes washed soon after instillation of the substance may be indicated Half a minute after instillation, the eyes of the animals are washed with water for 30 sec, using a volume and velocity of flow which will not cause injury

(5) **Observation period.** The duration of the observation period is at least 72 h, and should not be fixed rigidly, but should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed The observation period normally need not exceed 21 days after instillation

(6) **Clinical examination and scoring.** (i) The eyes should be examined at 1, 24, 48, and 72 h If there is no evidence of irritation at 72 h, the study may be ended Extended observation (e g at 7 and 21 days) may be necessary if there is persistent corneal involvement or other ocular irritation in order to determine the progress of the lesions and their reversibility or irreversibility In addition to the observations of the cornea, iris and conjunctivae, any other lesions which are noted should be recorded and reported. The grades of ocular reaction using the following table should be recorded at each examination

Grades for Ocular Lesions	
Cornea	
Opacity Degree of density (area most dense taken for reading) No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	*1
Easily discernible translucent area, details of iris slightly obscured	*2
Nacrous area, no details or iris visible, size of pupil barely discernible	*3
Opaque cornea, iris not discernible through the opacity	*4
Iris	
Normal	0
Markedly deepened rugae, congestion, swelling moderate circumcorneal hyperemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	*1
No reaction to light, hemorrhage, gross destruction (any or all of these)	*2
Conjunctivae	
Redness (refers to palpebral and bulbar conjunctivae, excluding cornea and iris)	
Blood vessels normal	0
Some blood vessels definitely hyperemic (injected)	1
Diffuse, crimson color, individual vessels not easily discernible	*2
Diffuse beefy red	*3
Chemosis (refers to lids and/or nictitating membranes)	
No swelling	0
Any swelling above normal (includes nictitating membranes)	1
Obvious swelling with partial eversion of lids	*2
Swelling with lids about half closed	*3
Swelling with lids more than half-closed	*4

*Starred figures indicate positive grades

(ii) Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observations at 24 h, the eyes of any or all rabbits may be further examined with the aid of fluorescein.

(iii) The grading of ocular responses is subject to various interpretations To promote harmonization and to assist testing laboratories and those involved in making and interpreting the observations, an illustrated guide in grading eye irritation should be used.

(g) **Data and reporting—(1) Data summary.** Data should be summarized in tabular form, showing for each individual animal the irritation scores at observation time up until reversal (nonpositive grades) or 21 days when the test is concluded, a description of the degree and nature of irritation, the presence of serious lesions and any effects other than ocular which were observed

(2) **Evaluation of the results.** The ocular irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed The individual scores do not represent an absolute standard for the irritant properties of a material They should be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations

(3) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i)Species, strain, sex, age, and source of test animal

(ii) Rationale for selection of species (if species is other than the species preferred

(iii) Tabulation of irritant/corrosive response data for each individual animal at each observation time point (e g 1, 24, 48, and 72 h until reversibility of lesions or termination of the test)

(iv) Description of any lesions observed

(v) Narrative description of the degree and nature of irritation or corrosion observed

(vi) Description of the method used to score the irritation at 1, 24, 48, and 72 h (e.g hand slit-lamp, biomicroscope, fluorescein stain)

(vii) Description of any nonocular effects noted

(viii) Description of any pre-test conditioning, including diet, quarantine, and treatment of disease

(ix) Description of caging conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animal

(x) Manufacturer, source, purity, and lot number of test substance

(xi) Physical nature, and, where appropriate, concentration and pH value for the test substance

(xii) Identification, composition, and characteristics of any vehicles (e g , diluents, suspending agents, emulsifiers, and anesthetics) or other materials used in administering the test substance

(xiii) A list of references cited in the body of the report, i.e , references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results

(h) **References.** The following references should be consulted for additional background information on this test guideline

(1) Buehler, E V and Newmann, E A A Comparison of Eye Irritation in Monkeys and Rabbits *Toxicology and Applied Pharmacology* 6 701–710 (1964)

(2) Draize, J H *Dermal Toxicity Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics* The Association of Food and Drug Officials of the United States (1959) 3rd printing 1975, pp 49–52

(3) Draize, J H et al Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes *Journal of Pharmacology and Experimental Therapeutics* 83 377–390 (1944)

(4) Loomis, T A *Essentials of Toxicology* Lea and Febiger, Philadelphia 3rd ed 1978 pp. 226–232

(5) Kay, J H and Calandra, J C , Interpretation of eye irritation tests *Journal of the Society of Cosmetic Chemists* 13 281–289 (1962)

(6) National Academy of Sciences *Principles and Procedures for Evaluating the Toxicity of Household Substances* A report propared by the Committee for the revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977)

(7) World Health Organization *Part I Environmental Health Criteria 6 Principles and Methods for Evaluating the Toxicity of Chemicals* World Health Organization, Geneva (1978)

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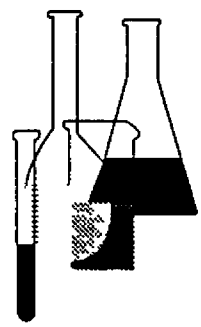
Health Effects Test Guidelines

OPPTS 870.2500
Acute Dermal Irritation



INTRODUCTION

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OPPTS 870 2500 Acute dermal irritation

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 798 4470 Primary Dermal Irritation, OPP 81–5 Primary Dermal Irritation (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 404 Acute Dermal Irritation/ Corrosion

(b) **Purpose.** Determination of the irritant and/or corrosive effects on skin of mammals is useful in the assessment and evaluation of the toxic characteristics of a substance where exposure by the dermal route is likely Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance Data on primary dermal irritation are required by 40 CFR part 158 to support the registration of each manufacturing-use product and end-use product. See specifically § § 158 50 and 158 340 to determine whether these data must be submitted

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

Dermal corrosion is the production of irreversible tissue damage in the skin following the application of the test substance.

Dermal irritation is the production of reversible inflammatory changes in the skin following the application of a test substance.

Pharmacological effect means any chemically induced physiological changes in the test animal

Target organ means any organ of a test animal showing evidence of an effect of chemical treatment

(d) **Principle of the test methods.** (1) The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control (except when severe irritation/corrosion is suspected and the stepwise procedure is used (see paragraph (f)(1)(iii) of this guideline)) The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 14 days

(2) When testing solids (which may be pulverized if considered necessary), the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account Liquid test substances are generally used undiluted

(e) **Initial considerations.** (1) Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less, or 11 5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties

(2) It is unnecessary to test materials which have been shown to be highly toxic (LD_{50} less than 200 mg/kg) by the dermal route or have been shown not to produce irritation of the skin at the limit test dose level of 2000 mg/kg body weight

(3) It may not be necessary to test *in vivo* materials for which corrosive properties are predicted on the basis of results from well validated and accepted *in vitro* tests If an *in vitro* test is performed before the *in vivo* test, a description or reference to the test, including details of the procedure, must be given together with results obtained with the test and reference substances

(4) It may not be necessary to test materials for which corrosive potential is predicted from structure-activity relationships

(f) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection

(ii) **Number of animals.** At least three healthy adult animals (either sex) should be used unless justification/reasoning for using fewer animals is provided. It is recommended that a stepwise procedure be used to expose one animal, followed by additional animals to clarify equivocal responses.

(iii) **Stepwise exposure of animals** A single rabbit may be used if it is suspected that the test material might produce severe irritation/ corrosion. Three test patches are applied concurrently or sequentially to the animal. The first patch is removed after 3 min If no serious skin reaction is observed, the second patch is removed after 1 hour If observations indicate that exposure can be continued humanely, the third patch is removed after 4 hours and the responses graded If a corrosive effect is observed after either 3 min or 1 hour of exposure, the test is immediately terminated by removal of the remaining patches If a corrosive effect is observed after an exposure of up to 4 hours, then further animal testing is not required If no corrosive effect is observed in one animal after a 4–hour exposure, the test is completed using two additional animals, each with one patch

only, for an exposure period of 4 hours If it is expected that the test substance will not produce severe irritancy or corrosion, the test may be started using three animals, each receiving one patch for an exposure period of 4 hours

(2) **Control animals.** Separate animals are not recommended for an untreated control group Adjacent areas of untreated skin of each animal may serve as a control for the test

(3) **Dose level.** A dose of 0 5 mL of liquid or 500 mg of solid or semisolid is applied to the test site

(4) **Preparation of test area.** Approximately 24 h before the test, fur should be removed from the test area by clipping or shaving from the dorsal area of the trunk of the animals Care should be taken to avoid abrading the skin Only animals with healthy intact skin should be used

(5) **Application of the test substance.** (i) The recommended exposure duration is normally 4 hours unless corrosion is observed (see paragraph (f)(1)(iii) of this guideline) Longer exposure may be indicated under certain conditions (e g expected pattern of human use and exposure) At the end of the exposure period, residual test substance should generally be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis

(ii) When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should by taken into account If a vehicle is used, it should not alter the absorption, distribution, metabolism, retention or the chemical properties of the test substance nor should it enhance, reduce, or alter its toxic characteristics Although water or saline is the preferred agent to be used for moistening dry test materials, other agents may be used providing the use is justified Acceptable alternatives include gum arabic, ethanol and water, carboxymethyl cellulose, polyethylene glycol, glycerol, vegetable oil, and mineral oil

(iii) The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with nonirritating tape In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and apply that to the skin The patch should be loosely held in contact with the skin by means of a suitable semiocclusive dressing for the duration of the exposure period Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented

(6) **Observation period.** The duration of the observation period need not be rigidly fixed It should be sufficient to fully evaluate the reversibility or irreversibility of the effects observed It need not exceed 14 days after application

(7) **Clinical examination and scoring.** (i) After removal of the patch, animals should be examined for signs of erythema and edema and the responses scored within 30–60 min, and at 24, 48, and 72 hours after patch removal

(ii) Dermal irritation should be scored and recorded according to the grades in the following Table 1 Further observations may be needed, as necessary, to establish reversibility In addition to the observation of irritation, any lesions and other toxic effects should be fully described

Table 1 —Evaluation of Skin Reaction

	Value
Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible	4
Edema Formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure	4
Maximum possible	4

(g) **Data and reporting**—(1) **Data summary.** Data should be summarized in tabular form, showing for each individual animal the irritation scores for erythema and edema at 30 to 60 min, and 24, 48, and 72 hours after patch removal, any other dermal lesions, a description of the degree and nature of irritation, corrosion and reversibility, and any other toxic effects observed

(2) **Evaluation of results.** The dermal irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed The individual scores do not represent an absolute standard for the irritant properties of a material They should be viewed as reference values which are only meaningful when supported by a full description and evaluation of the observations

(3) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported·

(i) Species, strain, sex, age and source of test animal

(ii) Rationale for selection of species (if species is other than the species preferred or required by OPP's toxicology data requirements for pesticide registration)

(iii) Tabulation of erythema and edema data and any other dermal lesions/responses for each individual animal at each observation time point (e g 30–60 minutes and 24, 48, and 72 hours until end of test/reversibility)

(iv) Description of any lesions observed

(v) Narrative description of the degree and nature of irritation or corrosion observed

(vi) Description of any systemic effects observed

(vii) Description of any pre-test conditioning, including diet, quarantine and treatment of disease

(viii) Description of caging conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animal.

(ix) Manufacturer, source, purity, and lot number of test substance.

(x) Physical nature, and, where appropriate, concentration and pH value for the test substance

(xi) Identification and composition of any vehicles (e g , diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance

(xii) A list of references cited in the body of the report, i e , references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results

(h) **References.** The following references should be consulted for additional background information on this test guideline

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United States
Environmental Protection
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Prevention Pesticides
and Toxic Substances
(7101)

EPA 712-C-98-197
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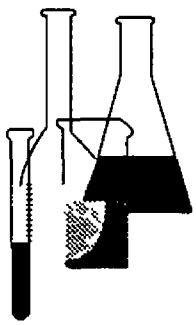
Health Effects Test Guidelines

OPPTS 870.2600
Skin Sensitization



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.2600 Skin sensitization.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are the OPPT 40 CFR 798 4100 Dermal Sensitization, OPP 81–6 Dermal Sensitization (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 406 Skin Sensitization

(b) **Purpose.** Determination of the potential to cause or elicit skin-sensitization reactions (allergic contact dermatitis) is an important element in evaluating a substance's toxicity Information derived from skin-sensitization tests serves to identify possible hazards to a population exposed repeatedly to a test substance The test selected should identify substances with significant allergenic potential and minimize false negative results

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Challenge exposure is an experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject will react in a hypersensitive manner

Induction exposure is an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state

Induction period is a period of a least 1 week following an induction exposure during which a hypersensitive state may develop

Skin sensitization (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance In the human, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these In other species, the reactions may differ and only erythema and edema may be seen

(d) **Principle of the test method.** Following initial exposure to a test substance, the animals are subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction with that of the initial induction exposure The test animals are initially exposed to the test substance by intradermal and/or epidermal application (induction exposure) Following a rest period of 10 to 14 days (the induction period), during which an immune response may develop, the

animals are exposed to a challenge dose The extent and degree of skin reaction to the challenge exposure is compared with that demonstrated by control animals that undergo sham treatment during induction and then receive the challenge exposure

(e) **Test procedures.** (1) Any of the following test methods is considered to be acceptable

(i) Buehler test

(ii) Guinea-pig maximization test (GPMT)

(iii) Other.

(A) Open epicutaneous test

(B) Maurer optimization test

(C) Split adjuvant technique

(D) Freund's complete adjuvant test

(E) Draize sensitization test

(2) The GPMT of Magnusson and Kligman, which uses adjuvant, and the nonadjuvant Buehler test are given preference over other methods. Although strong preference is given to either the Buehler test or the GPMT, it is recognized that other tests may give useful results If other tests are used, the tester should provide justification/reasoning for their use, methods and protocols must be provided, and each test should include a positive and a negative control group

(f) **Screening tests.** The mouse ear swelling test (MEST) (see paragraphs (i)(9), (i)(10), (i)(11), and (i)(12) of this guideline) or the local (auricular) lymph node assay (LLNA) (see paragraphs (i)(13), (i)(14), (i)(15), and (i)(16) of this guideline) in the mouse may be used as screening tests to detect moderate to strong sensitizers. If a positive result is seen in either assay, the test substance may be designated a potential sensitizer, and it may not be necessary to conduct a further test in guinea pigs If the LLNA or MEST does not indicate sensitization, the test substance should not be designated a nonsensitizer without confirmation in an accepted test using guinea pigs

(g) **Animal selection—(1) Species and strain.** The young adult guinea pig is preferred Commonly used laboratory strains should be employed If other species are used, the tester should provide justification/reasoning for their selection

(2) **Housing and feeding.** The temperature of the experimental animal room should be 20±3 °C with the relative humidity 30–70 percent. Where the lighting is artificial, the sequence should be 12 h light/

12 h dark Conventional laboratory diets may be used with an unlimited supply of drinking water It is essential that guinea pigs receive an adequate amount of ascorbic acid

(3) **Number and sex.** The number and sex will depend on the method chosen Either sex may be used in the Buehler test and the GPMT If females are used, they should be nulliparous and not pregnant The Buehler test recommends using a minimum of 20 animals in the treatment and at least 10 as controls At least 10 animals in the treatment group and 5 in the control group should be used with the GPMT, with the stipulation that if it is not possible to conclude that the test substance is a sensitizer after using fewer than 20 test and 10 control guinea pigs, the testing of additional animals to give a total of at least 20 test and 10 control animals is strongly recommended

(4) **Control animals.** (i) The sensitivity and reliability of the experimental technique used should be assessed every 6 months in naive animals by the use of positive control substances known to have mild-to-moderate skin-sensitizing properties In a properly conducted test, a response of at least 30 percent in an adjuvant test and at least 15 percent in a nonadjuvant test should be expected for mild-moderate sensitizers Preferred substances are hexylcinnamic aldehyde (CAS No 101–86–0), mercaptobenzothiazole (CAS No 149–30–4), benzocaine (CAS No 94–09–7), dinitro-chloro-benzene (CAS No 97–00–7), or DER 331 epoxy resin There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used

(ii) Depending upon the test selected, animals may be used as their own controls, but usually there will be a separate group of sham-treated animals that are exposed to the test substance only after the induction period, whose reactions are compared to those of the animals that have received both induction and challenge exposures Control groups which provide the best design should be used Some cases may best be served by both naive and vehicle control groups

(5) **Dose levels.** The dose level will depend on the test method selected In the Buehler test, the concentration of the induction dose should be high enough to cause mild irritation, and the challenge dose should use the highest non-irritating concentration In the GPMT, the concentration of the induction dose should be well tolerated systemically, and should be high enough to cause mild-to-moderate skin irritation, the GPMT challenge dose should use the highest non-irritating concentration

(6) **Observation of animals.** (i) Skin reactions should be graded and recorded after the challenge exposures at the time specified by the methodology selected This is usually at 24 and 48 hours Additional notations should be made as necessary to fully describe unusual responses

(ii) Regardless of the test method selected, initial and terminal body weights should be taken and recorded

(7) **Procedures.** The procedures to be used are those described by the test method chosen Brief summaries are given here, but the tester should refer to the original literature for more complete guidance on conducting the Buehler test (under paragraphs (i)(1), (i)(2), (i)(3), and (i)(4) of this guideline) or the GPMT (under paragraphs (i)(5), (i)(6), (i)(7), and (i)(8) of this guideline)

(i) The Buehler test uses topical administration via a closed patch on days 0, 6–8, and 13–15 for induction, with topical challenge of the untreated flank for 6 hours on day 27–28 Readings are made approximately 24 hours after removing the challenge patch, and again 24 hours after that If the results are equivocal, the animals may be rechallenged one week later, using either the original control group or a new control group for comparison See paragraphs (i)(1), (i)(2), (i)(3), and (i)(4) of this guideline

(ii) The GPMT uses intradermal injection with and without Freund's complete adjuvant (FCA) for induction, followed on days 5–8 by topical irritation/induction, followed by topical challenge for 24 hours on day 20–22 Readings are made approximately 24 hours after removal of the challenge dose, and again after another 24 hours As with the Buehler test, if the results are equivocal, the animals may be rechallenged 1 week later If only 10 animals were used initially and gave equivocal results, the use of an additional 10 experimental and 5 control animals is strongly recommended. See paragraphs (i)(5), (i)(6), (i)(7), and (i)(8) of this guideline

(iii) Blind reading of both test and control animals is recommended.

(iv) Removal of the test material should be accomplished with water or an appropriate solvent, without altering the existing response or the integrity of the epidermis

(v) Hair is removed from the site of application by clipping, shaving, or possibly by depilation, depending on the test selected.

(h) **Data and reporting.** Data should be summarized in tabular form, showing for each individual animal the skin reaction, results of the induction exposure, and the challenge exposure at times indicated by the method chosen. As a minimum, the erythema and edema should be graded and any unusual finding should be recorded

(1) **Evaluation of the results.** The evaluation of results will provide information on the proportion of each group that became sensitized and the extent (slight, moderate, severe) of the sensitization reaction in each individual animal

(2) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 158 (for pesticides) and 40 CFR part 792, subpart J (for toxic substances), the following specific information should be reported

(i) A description of the method used and the commonly accepted name

(ii) Information on the positive control study, including the positive control substance used, the method used, and the time conducted

(iii) The number, species, strain, age, source, and sex of the test animals

(iv) Individual weights of the animals at the start of the test and at the conclusion of the test.

(v) A brief description of the grading system

(vi) Each reading made on each individual animal

(vii) The chemical identification and relevant physicochemical properties of the test substance

(viii) The vehicles used for induction and challenge, and justification for their use, if other than water or physiological saline Any material that might reasonably be expected to react with or enhance or retard absorption of the test substance should be reported

(ix) The total amount of test substance applied for induction and challenge, and the technique of application in each case

(x) Description of any pre-test conditioning, including diet, quarantine and treatment of disease

(xi) Description of caging conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animal.

(xii) Histopathological findings, if any

(xiii) Discussion of results

(xiv) Manufacturer, source, purity, and lot number of test substance

(xv) Physical nature, and, where appropriate, concentration and pH value for the test substance

(xvi) A list of references cited in the body of the report, i e , references to any published literature used in developing the test protocol,

performing the testing, making and interpreting observations, and compiling and evaluating the results

(i) **References.** The following references should be consulted for additional background information on this test guideline

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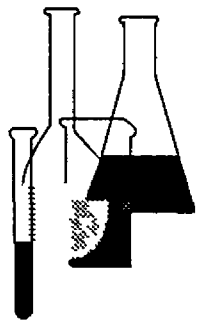
Health Effects Test Guidelines

OPPTS 870.3100
90–Day Oral Toxicity in Rodents



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

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OPPTS 870.3100 90-Day oral toxicity in rodents.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 798 2650 Oral Toxicity, OPP 82–1 90–Day Oral—Two Species, Rodent and Nonrodent, and OECD 408 Subchronic Oral Toxicity—Rodent 90–Day.

(b) **Purpose.** In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing The subchronic oral study has been designed to permit the determination of the no-observed-effect level (NOEL) and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days This study is not capable of determining those effects that have a long latency period for development (e g , carcinogenicity and life shortening) Extrapolation from the results of this study to humans is valid only to a limited degree However, it can useful in providing information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time, such as target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure

(c) **Definitions.** The definitions in section 3 of the Toxic Substance Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline

Cumulative toxicity is the adverse effects of repeated dosesoccurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissue.

Dose in a subchronic oral study is the amount of test substance administered daily via the oral route (gavage, drinking water or diet) for a period of 90 days Dose is expressed as weight of the test substance (grams, milligrams) per unit body weight (BW) of test animal (milligram per kilogram) or as weight of the test substance in parts per million in food or drinking water per day

No-observed-effect-level (NOEL) is the maximum dose used in a study which produces no adverse effects The NOEL is usually expressed in terms of the weight of a test substance given daily per unit weight of test animal (milligrams per kilogram per day)

Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by

the oral route for a part (approximately 10 percent) of the test animal's life span.

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects or if toxic effects would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary

(e) **Test procedures—(1) Animal selection—(i) Species and strain.** A variety of rodent species may be used, although the rat is the preferred species Commonly used laboratory strains should be employed

(ii) **Age/weight.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization

(B) Dosing of rodents should generally begin no later than 8–9 weeks of age

(C) At the commencement of the study the weight variation of animals used should be within 20 percent of the mean weight for each sex.

(iii) **Sex.** Equal numbers of animals of each sex should be used at each dose level, and the females should be nulliparous and nonpregnant.

(iv) **Numbers.** (A) At least 20 rodents (10 males and 10 females) at each dose level

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required

(D) Each animal should be assigned a unique identification number Dead animals, their preserved organs and tissues, and microscopic slides should be identified by reference to the animal's unique number.

(v) **Husbandry.** (A) Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal The biological properties of the test substance or toxic effects (e g , morbidity, excitability) may indicate a need for individual caging.

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C

(C) The relative humidity of the experimental animal rooms should be 50 ± 20 percent

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark

(E) Control and test animals should be fed from the same batch and lot The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least five days is recommended

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle If a vehicle or diluent is needed, the vehicle should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution in oil and then solution in other vehicles

(ii) If possible, one lot of the test substance tested should be used throughout the duration of the study and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound and, if technically feasible, the names and quantities of contaminants and impurities

(iii) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture

(3) **Control groups.** A concurrent control group is required This group should be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required

(4) **Satellite group.** A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days In addition, a control group of 20 animals (10 animals of each sex) should be added to the satellite study

(5) **Dose levels and dose selection.** (i) In subchronic toxicity tests, it is desirable to determine a dose-response relationship as well as a NOEL Therefore, at least three dose levels plus a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest dose level) should be used Doses should be spaced appropriately to produce test groups with a range of toxic effects The data should be sufficient to produce a dose-response curve

(ii) The highest dose level should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects

(iv) The lowest dose level should produce no evidence of toxicity

(6) **Administration of the test substance.** (i) If the test substance is administered by gavage, the animals are dosed with the test substance on a 7–day per week basis for a period of at least 90 days However, based primarily on practical considerations, dosing by gavage on a 5–day per week basis is acceptable If the test substance is administered in the drinking water, or mixed in the diet, then exposure should be on a 7–day per week basis

(ii) All animals should be dosed by the same method during the entire experimental period

(iii) For substances of low toxicity, it is important to ensure that when administered in the diet the quantities of the test substance involved do not interfere with normal nutrition When the test substance is administered in the diet, either a constant dietary concentration (parts per million) or a constant dose level in terms of body weight should be used, the alternative used should be specified

(iv) For a substance administered by gavage, the dose should be given at approximately the same time each day, and adjusted at intervals (weekly or biweekly) to maintain a constant dose level in terms of body weight

(7) **Observation period.** (i) The animals should be observed for a period of 90 days

(ii) Animals in the satellite group (if used) scheduled for follow-up observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects

(8) **Observation of animals.** (i) Observations should be made at least twice each day for morbidity and mortality Appropriate actions should be taken to minimize loss of animals to the study (e g , necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals) General clinical observations should be made at least once a day, preferably at the same time each day, taking into consideration the, peak period of anticipated effects after dosing The clinical condition of the animal should be recorded

(ii) A careful clinical examination should be made at least once prior to the initiation of treatment (to allow for within subject comparisons) and once weekly during treatment in all animals These observations should be made outside the home cage, preferably in a standard arena, and at similar times on each occasion Effort should be made to ensure that variations in the observation conditions are minimal Observations should be detailed and carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e g., lacrimation, piloerection, pupil size, unusual respiratory pattern) Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e g , excessive grooming, repetitive circling) or bizarre behavior (e g , self-mutilation, walking backwards) should be recorded

(iii) Once, near the end of the exposure period and in any case not earlier than in week 11, assessment of motor activity, grip strength, and sensory reactivity to stimuli of different types (e g , visual, auditory, and proprioceptive stimuli) should be conducted Further details of the procedures that could be followed are described in the references listed under paragraphs (h)(2), (h)(5), (h)(6), (h)(7), (h)(8), and (h)(11) of this guideline

(iv) Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits

(v) Exceptionally, functional observations may be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with functional test performance

(vi) Measurements of food consumption and water consumption, if drinking water is the exposure route, should be made weekly

(vii) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death

(viii) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible

(ix) At termination, all survivors in the treatment and control groups should be sacrificed

(9) **Clinical pathology.** Hematology and clinical chemistry examinations should be made on all animals, including controls, of each sex in each group The hematology and clinical chemistry parameters should be examined at terminal sacrifice at the end of the study Overnight fasting of the animals prior to blood sampling is recommended Overall, there is a need for a flexible approach in the measures examined, depending on the observed or expected effects from a chemical, and in the frequency of measures, depending on the duration of potential chemical exposures

(i) Hematology The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time

(ii) Clinical chemistry (A) Parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity

(B) The recommended clinical chemistry determinations are potassium, sodium, glucose, total cholesterol, urea nitrogen, creatinine, total protein and albumin More than 2 hepatic enzymes, (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, or gamma glutamyl transpeptidase) should also be measured Measurements of additional enzymes (of hepatic or other origin) and bile acids, may also be useful

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include calcium, phosphorus, fasting triglycerides, hormones, methemoglobin, and cholinesterases

(iii) Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collec-

tion appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells

(10) **Ophthalmological examination.** Ophthalmological examinations using an ophthalmoscope or an equivalent device should be made on all animals prior to the administration of the test substance and on all high dose and control groups at termination If changes in the eyes are detected, all animals in the other dose groups should be examined

(11) **Gross necropsy.** (i) All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents

(ii) The liver, kidneys, adrenals, testes, epididymides, ovaries, uterus, thymus, spleen, brain, and heart should be trimmed and weighed wet, as soon as possible after dissection

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder (when present)

(B) Nervous system—brain (including sections of medulla/pons, cerebellum and cerebrum), pituitary, peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle), spinal cord (three levels: cervical, mid-thoracic and lumbar), eyes (retina, optic nerve)

(C) Glandular system—adrenals, parathyroid, thyroid.

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose

(E) Cardiovascular/hemopoietic system—aorta, heart, bone marrow (and/or fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen, thymus

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland

(G) Others—all gross lesions and masses, skin

(13) **Histopathology.** (i) The following histopathology should be performed

(A) Full histopathology on the organs and tissues, listed under paragraph (e)(12)(iii) of this guideline, of all rodents in the control and high dose groups, and all rodents that died or were killed during the study

(B) All gross lesions in all animals

(C) Target tissues in all animals

(D) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion

(ii) When applicable, all observed results, qualitative and quantitative, should be evaluated by an appropriate and generally accepted statistical method. Any generally accepted statistical methods may be used, the statistical methods, including significance criteria, should be selected during the design of the study

(2) **Evaluation of study results.** The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects A properly conducted subchronic test should provide a satisfactory estimation of a NOEL It also can indicate the need for an additional longer-term study and provide information on the selection of dose levels

(3) **Test report.** In addition to reporting requirements specified under EPA Good Laboratory Practice Standards at 40 CFR part 792, subpart J and 40 CFR part 160, and the OECD principles of GLP (ISBN 92–64–12367–9), the following specific information should be reported·

(i) Test substance characterization should include

(A) Chemical identification

(B) Lot or batch number

(C) Physical properties

(D) Purity/impurities

(ii) Identification and composition of any vehicle used

(iii) Test system should contain data on

(A) Species and strain of animals used and rationale for selection if other than that recommended

(B) Age including body weight data and sex

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(D) Identification of animal diet

(E) Acclimation period

(iv) Test procedure should include the following data

(A) Method of randomization used

(B) Full description of experimental design and procedure

(C) Dose regimen including levels, methods, and volume

(v) Test results should include

(A) Group animal data Tabulation of toxic response data by species, strain, sex and exposure level for

(*1*) Number of animals exposed

(*2*) Number of animals showing signs of toxicity

(*3*) Number of animals dying

(B) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(*1*) Date of death during the study or whether animals survived to termination

(*2*) Date of observation of each abnormal sign and its subsequent course

(*3*) Body weight data

(*4*) Feed and water (if collected) consumption data

(5) Achieved dose (mg/kg/day) as a time-weighted average if the test substance is administered in the diet or drinking water

(*6*) Results of ophthalmological examination

(*7*) Results of hematological tests performed

(*8*) Results of clinical chemistry tests performed

(*9*) Results of urinalysis, if performed

(*10*) Necropsy findings, including absolute and relative (to body weight) organ weight data

(*11*) Detailed description of all histopathological findings.

(*12*) Statistical treatment of results, where appropriate.

(g) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment The study must be conducted in compliance with GLP regulations

(h) **References.** The following references should be consulted for additional background information on this test guideline

(1) Boyd, E M Chapter 14 Pilot Studies, 15 Uniposal Clinical Parameters, 16 Uniposal Autopsy Parameters Predictive Toxicometrics Williams and Wilkins, Baltimore (1972)

(2) Crofton K M., Howard J L , Moser V C , Gill M.W , Leiter L.W , Tilson H A., MacPhail, R C Interlaboratory Comparison of Motor Activity Experiments Implication for Neurotoxicological Assesments *Neurotoxicol Teratol* 13, 599-609 (1991)

(3) Fitzhugh, O G Subacute Toxicity, Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp 260935.

(4) Food Safety Council Subchronic Toxicity Studies, Proposed System for Food Safety Assessment (Columbia· Food Safety Council, 1978) pp 830996

(5) Gad S C A Neuromuscular Screen for Use in Industrial Toxicology *Journal of Toxicology and Environmental Health* 9, 691-704 (1982)

(6) International Programme on Chemical Safety Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals Environmental Health Criteria Document No. 60 (1986)

(7) Meyer O A , Tilson H A , Byrd W C , Riley M T A Method for the Routine Assessment of Fore- and Hind-Limb Grip Strength of Rats and Mice *Neurobehav Toxicol* 1, 233-236 (1979)

(8) Moser V C , McDaniel K M , Phillips P M Rat Strain and Stock Comparisons using a Functional Observational Battery Baseline Values and Effects of Amitraz *Toxicol Appl Pharmacol* 108, 267-283 (1991)

(9) National Academy of Sciences Principles and Procedures for Evaluating the Toxicity of Household Substances, A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977)

(10) Organization for Economic Cooperation and Development. OECD Guidelines for Testing of Chemicals Guideline 408 Subchronic Oral Toxicity-Rodent 90-day Study, Adopted May 12, 1981

(11) Tupper, D E , Wallace R B Utility of the Neurologic Examination in Rats Acta *Neurobiol Exp* 40, 999-1003 (1980)

(12) United States Environmental Protection Agency Office of Testing and Evaluation Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules. 40 CFR Part 772. Standard for Development of Test Data Subpart D FEDERAL REGISTER Vol 44, pp 27350-27362

(13) Weingand K , Brown G , Hall R. et al Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies *Fundam & Appl Toxicol* 29 198-201 (1996)

(14) World Health Organization Guidelines for Evaluation of Drugs for Use in Man, WHO Technical Report Series No 563. (Geneva: World Health Organization, 1975)

(15) World Health Organization Part I Environmental Health Criteria 6, Principles and Methods for Evaluating the Toxicity of Chemicals (Geneva· World Health Organization, 1978)

(16) World Health Organization Principles for Pre-Clinical Testing of Drug Safety, WHO Technical Report Series No 341 (Geneva World Health Organization, 1966)

United States
Environmental Protection
Agency

Prevention Pesticides
and Toxic Substances
(7101)

EPA 712-C-98-200
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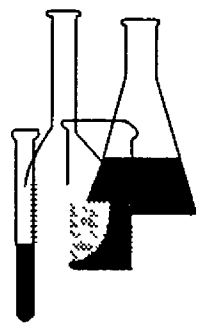
Health Effects Test Guidelines

OPPTS 870.3150
90-Day Oral Toxicity in Nonrodents



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in title 40, chapter I, subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S. Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*).

Final Guideline Release: This guideline is available from the U.S Government Printing Office, Washington, DC 20402 on disks or paper copies call (202) 512–0132 This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http //www epa.gov/epahome/research htm) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines "

OPPTS 870.3150 90-day oral toxicity in nonrodents.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline is OPP 82–1 90–Day Oral—Two Species, Rodent and Nonrodent (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 409 Subchronic Oral Toxicity—Nonrodent 90–Day

(b) **Purpose.** The determination of subchronic oral toxicity in the assessment and evaluation of the toxic characteristics of a chemical may be carried out after initial information on toxicity has been obtained by acute testing The subchronic oral study has been designed to permit the determination of the no-observed-effect level (NOEL) and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days The test is not capable of determining those effects that have a long latency period for development (e g carcinogenicity and life shortening) Extrapolation from the results of this study to humans is valid only to a limited degree However, it can be useful in providing information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time It provides information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline.

Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissue

Dose in an oral subchronic study is the amount of test substance administered daily via the oral route (gavage, capsules, diet or drinking water) for 90 days Dose is expressed as weight of test substance (grams, milligrams) per unit weight of test animal (e.g. milligrams per kilogram), or as weight of test substance per unit weight of food or drinking water per day

No-observed-effect-level (NOEL) is the maximum dose used in a test which produces no observed adverse effects A NOEL is expressed in terms of the weight of a substance given daily per unit weight of test animal (milligrams per kilogram)

Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral route for a part of the test animal s life span

Target organ is any organ of a test animal showing evidence of an effect induced by the test substance

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (BW) (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, a full study using three dose levels might not be necessary

(e) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** A mammalian nonrodent species should be used for testing The commonly used nonrodent species is the dog, preferably of a defined breed, the beagle is frequently used If other mammalian species are used, the tester should provide justification/reasoning for his or her selection.

(ii) **Age/weight.** (A) Young adult animals should be used.

(B) In the case of the dog, dosing should commence after acclimation, preferably at 4 to 6 months and not later than 9 months of age.

(C)At the commencement of the study the weight variation of animals used should be within 20 percent of the mean weight for each sex.

(iii) **Sex.** (A) Equal numbers of animals of each sex should be used at each dose level

(B) The females should be nulliparous and nonpregnant

(iv) **Numbers.** (A) At least eight animals (four females and four males) should be used at each dose level

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required

(D) Each animal should be assigned a unique identification number. Dead animals, their preserved organs and tissues and microscopic slides should be identified by reference to the animal's unique number

(v) **Husbandry.** (A) Caging and environmental conditions should be appropriate to the nonrodent species However, it is recommended that dogs are housed individually The number of animals per cage must not interfere with a clear observation of each animal

(B) For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water The choice of diet may be influenced by the need to ensure a suitable admixture of the test substance when administered by this method

(C) Control and test animals should be fed from the same batch and lot The feed should be analyzed to assure adequacy of nutritional requirements of the species tested for impurities that might influence the outcome of the test For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(D) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least 5 days is recommended

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle If a vehicle or diluent is needed, the vehicle should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance It is recommended that whenever possible the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then solution in other vehicles

(ii) If possible, one lot of the test substance tested should be used throughout the duration of the study and the research sample should be stored under conditions that maintain its purity and stability Prior to the initiation of the study, there should be characterization of the test substance, including the purity of the test compound and, if technically feasible, the names and quantities of contaminants and impurities

(iii) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study. Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(3) **Control groups.** A concurrent control group is required This group should be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required

(4) **Satellite group.** A satellite group of eight animals (four animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days In addition, a control group of 8 animals (4 animals per sex) should be added to the satellite study

(5) **Dose levels and dose selection.** (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as a NOEL Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve

(ii) The highest dose level should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects

(iv) The lowest dose level should not produce any evidence of toxicity.

(6) **Administration of the test substance.** (i) The test substance may be administered in the diet, drinking water, by gavage or in capsules Ideally, if the test substance is administered by gavage or in capsules, the animals should be dosed with the test material on a 7-day per week basis for a period of at least 90 days However, based primarily on practical considerations, dosing by gavage or with capsules on a 5-day per week basis is acceptable If the test substance is administered in the drinking water or mixed in the diet, then exposure should be on a 7-day per week basis.

(ii) All animals should be dosed by the same method during the entire experimental period

(iii) For substances of low toxicity, it is important to ensure that when administered in the diet the quantities of the test substance involved do not interfere with normal nutrition When the test substance is administered in the diet, either a constant dietary concentration (parts per million) or a constant dose level in terms of the body weight of the animals should be used, the alternative used should be specified

(iv) For a substance administered by gavage or capsules, the dose should be given at approximately the same time each day, and adjusted at intervals (weekly or biweekly) to maintain a constant dose level in terms of animal body weight

(7) **Observation period.** (i) Duration of observation should be for at least 90 days

(ii) Animals in the satellite group (if used) scheduled for follow-up observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects

(8) **Observation of animals.** (i) Each animal should be observed twice daily for morbidity and mortality Appropriate actions should be taken to minimize loss of animals to the study (e g , necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals) General clinical observations should be made at least once a day, preferably at the same time each day, taking into consideration the peak period of anticipated effects after dosing The clinical condition of the animal should be recorded

(ii) A careful clinical examination should be made prior to the initiation of treatment and at least once weekly during treatment Detailed observations should be made on all animals These observations should be made, where practical, outside the home cage in a standard arena and preferably at similar times on each occasion Effort should be made to ensure that variations in the observation conditions are minimal Signs of toxicity should be carefully recorded, including time of onset, degree and duration. Observations should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, and autonomic activity (e g , lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in level of activity, gait, posture, altered strength, and response to handling as well as the presence of clonic or tonic movements, stereotypies (e g , excessive grooming, repetitive circling) or bizarre behavior (e g , self-mutilation) should be recorded

(iii) Measurements of feed consumption and water consumption, when drinking water is the exposure route, should be made weekly

(iv) Animals should be weighed shortly before the test substance is administered and weekly during the treatment period

(v) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible

(vi) At the end of the 90-day period all survivors in the nonsatellite control and treatment groups should be sacrificed

(9) **Clinical pathology.** Hematology and clinical chemistry examinations should be made on all animals, including controls, of each sex in each group The hematology and clinical chemistry parameters should be examined prior to treatment, either at monthly intervals or midway through the treatment period, and at the end of the treatment period on all groups of animals, including concurrent controls Overnight fasting of the animals

prior to blood sampling is recommended Overall, there is a need for a flexible approach in the measures examined, depending on the observed or expected effects from a chemical, and in the frequency of measures, depending on the duration of potential chemical exposures

(i) Hematology The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time and activated partial thromboplastin time

(ii) Clinical chemistry (A) Clinical biochemistry test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity

(B) The recommended clinical chemistry determinations are potassium, sodium, calcium, phosphorus, chloride, glucose, total cholesterol, urea nitrogen, creatinine, total protein, total bilirubin, and albumin Suggested hepatic enzymes include alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, and gamma glutamyl transpeptidase Measurements of addtional enzymes (of hepatic or other origin) and bile acids may also be useful

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include fasting triglycerides, hormones, methemoglobin, and cholinesterases

(iii) Urinalysis should be performed prior to treatment, midway through treatment and at the end of the study using timed urine collection Urinalysis determinations include· appearance, volume, osmolality or specific gravity, pH, protein, glucose, and blood/blood cells

(10) **Ophthalmological examination.** Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made on all animals prior to the administration of the test substance and at termination of the study, preferably in all animals but at least the high dose and control groups If changes in the eyes are detected, all animals in the other dose groups should be examined

(11) **Gross necropsy.** (i) All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents

(ii) At least the liver (with gall bladder), kidneys, adrenals, testes, epididymides, ovaries, uterus thyroid (with parathyroid), thymus, spleen, brain, and heart should be weighed wet as soon as possible after dissection to avoid drying

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder

(B) Nervous system—brain (multiple sections, including cerebrum, cerebellum and medulla/pons), pituitary, peripheral nerve (sciatic or tibia, preferably in close proximity to the muscle), spinal cord (three levels, cervical, mid-thoracic and lumbar), eyes (retina, optic nerve)

(C) Glandular system—adrenals, parathyroid, thyroid

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose

(E) Cardiovascular/hematopoietic system—aorta, heart, bone marrow (and/or a fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen, thymus.

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland

(G) Other—all gross lesions and masses, skin

(12) **Histopathology.** The following histopathology should be performed.

(i) Full histopathology on the organs and tissues, listed in paragraph (e)(11)(iii) of this guideline, in at least all animals in the control- and high-dose groups. The examination should be extended to all animals in all dosage groups if treatment-related changes are observed in the high-dose group

(A) All gross lesions in all animals

(B) Target organs in all animals

(C) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated group

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion

(ii) When applicable, all numerical results should be evaluated by an appropriate and generally acceptable statistical method Any generally accepted statistical methods may be used, the statistical methods should be selected during the design of the study

(2) **Evaluation of the study results.** (i) The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects A properly conducted subchronic test should provide a satisfactory estimation of a NOEL It can also indicate the need for an additional longer-term study and provide information on selection of dose levels.

(3) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J (Good Laboratory Practice Standards), 40 CFR part 160 and the OECD Principles of GLP (ISBN 92–64–12367–9) the following specific information should be reported·

(i) Test substance characterization should include

(A) Chemical identification

(B) Lot or batch numbers

(C) Physical properties

(D) Purity/impurities

(ii) Identification and composition of any vehicle used

(iii) Test system should contain data on

(A) Species and strain of animals used and rationale for selection if other than that recommended

(B) Age, including body weight data and sex

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(D) Identification of animal diet

(E) Acclimation period

(iv) Test procedure should include the following data

(A) Method of randomization used

(B) Full description of experimental design and procedure

(C) Dose regime including levels, method, and volume

(v) Test results should include

(A) Group animal data Tabulation of toxic response data by species, strain, sex, and exposure level for·

(*1*) Number of animals exposed

(*2*) Number of animals showing signs of toxicity

(*3*) Number of animals dying

(B) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(*1*) Date of death during the study or whether animals survived to termination

(*2*) Date of observation of each abnormal sign and its subsequent course.

(*3*) Body weight data

(*4*) Feed and water (when collected) consumption data

(*5*) Achieved dose (mg/kg/day) as a time-weighted average if the test substance is administered in the diet or drinking water

(*6*) Ophthalmological examination data

(*7*) Hematological tests employed and all results

(*8*) Clinical biochemistry tests employed and all results

(*9*) Urinalysis tests employed and all results

(*10*) Necropsy findings, including absolute and relative (to body weight) organ weight data

(*11*) Detailed description of all histopathological findings

(*12*) Statistical treatment of results, where appropriate

(h) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment The study must be conducted in compliance with GLP regulations

(i) **References.** The following references should be consulted for additional background information on this test guideline

(1) Boyd, E M Chapter 14—Pilot Studies, 15—Uniposal Clinical Parameters, 16—Uniposal Autopsy Parameters, in Predictive Toxicometrics Williams and Wilkins, Baltimore, MD (1972)

(2) Fitzhugh, O G Subacute Toxicity, in Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp 26–35

(3) Food Safety Council Subchronic Toxicity Studies, in Proposed System for Food Safety Assessment. Food Safety Council, Columbia (1978) pp 83–96

(4) National Academy of Sciences Principles and Procedures for Evaluating the Toxicity of Household Substances, a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977)

(5) Weingand K , Brown G , Hall R et al Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies *Fundam. & Appl Toxicol.* 29 198–201 (1996)

(6) World Health Organization Part I Environmental Health Criteria 6, in Principles and Methods for Evaluating the Toxicity of Chemicals World Health Organization, Geneva (1978)

United States
Environmental Protection
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Prevention Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–201
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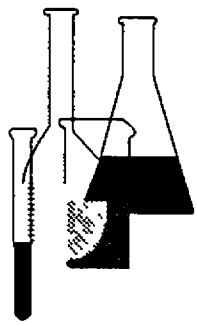
Health Effects Test Guidelines

OPPTS 870.3200
21/28-Day Dermal Toxicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in title 40, chapter I, subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

Final Guideline Release: This guideline is available from the U S Government Printing Office, Washington, DC 20402 on disks or paper copies call (202) 512–0132 This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http //www epa gov/epahome/research htm) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines "

OPPTS 870.3200 21/28-Day dermal toxicity.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are OPP 82–2 21-Day Dermal (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD guideline 410 Repeated Dose Dermal Toxicity 21–28 Day

(b) **Purpose.** A 21/28 day repeated dose dermal study will provide information on possible health hazards likely to arise from repeated dermal exposure to a test substance for a period of 21/28 days This study is not capable of determining those effects that have a long latency period for development (e g , carcinogenicity and life shortening) Extrapolation from the results of this study to humans is valid only to a limited degree. It can, however, provide useful information on the degree of percutaneous absorption, target organs, the possibilities of accumulation, and can be of use in selecting dose levels for longer-term studies and for establishing safety criteria for human exposure

(c) **Definitions.** The definitions in section 3 of the Toxic Substance Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline

Cumulative toxicity is the adverse effect of repeated doses occurring as a result of prolonged action or increased concentration of the administered test substance or its metabolites in susceptible tissues

Dose in a 21/28 day repeated dose dermal study is the amount of test substance applied daily to the skin for 21/28 days Dose is expressed as weight of the test substance (grams, milligrams), or as weight of the test substance per unit body weight of test animal (milligrams per kilogram), or as weight of test substance per unit of surface area (milligrams per square centimeter)

No-observed-effect level (NOEL) is the maximum dose used in a study which produces no adverse effects The NOEL level is expressed in terms of the weight of a test substance given daily per unit weight of test animal (milligrams per kilogram per day)

Repeated dose dermal study is used to embrace the toxic effects associated with repeated doses of a chemical over part of a life span of the test animal Dosing periods lying between a single dose and 10 percent of life span are often referred to as subacute The term subacute is semantically incorrect To distinguish such a dosing period from the classical sub-

chronic period. it may be described as short-term repeated dose study Study durations have been 14, 21, or 28 days

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this guideline, produces no observable toxic effects, or if toxic effects would not be expected based upon data on structurally related compounds, a full study using three dose levels might not be necessary

(e) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** A mammalian species should be used for testing The rat, rabbit, or guinea pig may be used. Commonly used laboratory strains should be employed If other mammalian species are used, the tester should provide justification/reasoning for their selection When a subchronic dermal study is conducted as a preliminary to a chronic study, the same species and strain should be used in both studies

(ii) **Age.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization

(B) Dosing should generally begin in guinea pigs between 5-6 weeks of age, in rats between 8-9 weeks of age, and in rabbits at least 12 weeks old

(C) At the commencement of the study, the weight variation of animals used should not exceed 20 percent of the mean weight for each sex.

(iii) **Sex.** Equal numbers of animals of each sex with healthy skin should be used at each dose level The females should be nulliparous and nonpregnant except for specially designed studies

(iv) **Numbers.** (A) For studies where the data will form the definitive basis for a risk assessment, e g , where a NOEL for risk assessment is needed, 10 animals/sex/dose will be needed For screening studies, 5 animals/sex/dose will generally be sufficient

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before completion of the study

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required

(D) Each animal must be assigned a unique identification number Dead animals, their preserved organs and tissues, and microscopic slides should be identified by reference to an animal's unique number

(v) **Husbandry.** (A) Animals should be housed in individual cages

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C for rodents and 20 ± 3 °C for rabbits

(C) The relative humidity of the experimental animal rooms should be 30 to 70 percent

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark

(E) Control and test animals should be fed from the same batch and lot The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least 5 days is recommended

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle If a vehicle or diluent is needed, the vehicle should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance. It is recommended that, whenever possible, the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then solution of other vehicles

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound and if technically feasible, the name and quantities of unknown contaminants and impurities.

(iii) If the test substance is dissolved or suspended in a vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture

(3) **Control groups.** A concurrent control group is required This group should be an untreated or sham-treated control group or, if a vehicle is used in the application of the test substance, a vehicle control group

If the toxic properties of the vehicle are not known or not available, both untreated/sham-treated and vehicle control groups are required

(4) **Satellite group.** A satellite group of 20 animals (10 animals per sex) may be treated with the high-dose level for 21/28 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of 14 days In addition a control group of 20 animals (10 animals of each sex) should be added to the satellite study

(5) **Dose levels and dose selection.** (i) For the repeated dose study, it is desirable to determine a dose-response relationship as well as a NOEL Therefore, at least three dose levels plus a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest-dose level) group should be used Doses should be spaced appropriately to produce test groups with a range of toxic effects The data should be sufficient to produce a dose-response curve

(ii) The highest-dose level should result in toxic effects but not produce severe skin irritation or an incidence of fatality which would prevent a meaningful evaluation If application of the test substance produces severe skin irritation, the concentration may be reduced, although this may result in a reduction in, or absence of, other toxic effects at the high-dose level. If the skin has been badly damaged early in the study, it may be necessary to terminate the study and undertake a new one at lower concentrations

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects

(iv) The lowest-dose level should not produce any evidence of toxic effects

(6) **Preparation of animal skin.** Fur should be clipped from not less than 10 percent of the body surface area shortly before testing for application of the test substance In order to dose approximately 10 percent of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half way down the flank on each side of the animal should be shaved. Shaving should be carried out approximately 24 hours before the test. Repeated clipping or shaving is usually needed at approximately weekly intervals When clipping or shaving the fur, care should be taken to avoid abrading the skin (which could alter its permeability)

(7) **Preparation of test substance.** (i) Liquid test substances are generally used undiluted, except as indicated in paragraph (e)(5)(ii) of this guideline.

(ii) Solids should be pulverized when possible The substance should be moistened sufficiently with water or, when necessary, a suitable vehicle

to ensure good contact with the skin When a vehicle is used, the influence of the vehicle on toxicity of, and penetration of the skin by, the test substance should be taken into account

(iii) The volume of application should be kept constant, e g less than 300 µL for the rat, different concentrations of test solution should be prepared for different dose levels

(8) **Administration of test substance.** (i) The duration of exposure should be at least for 21/28 days

(ii) The animals should be treated with test substance for at least 6 h/day on a 7-day per week basis However, based on practical considerations, application on a 5-day per week basis is acceptable Dosing should be conducted at approximately the same time each day

(iii) The test substance should be applied uniformly over the treatment site

(iv) The surface area covered may be less for highly toxic substances As much of the area should be covered with as thin and uniform a film as possible

(v) During the exposure period, the test substance should be held in contact with the skin with a, porous gauze dressing (less than or equal to 8 ply) The test site should be further covered with nonirritating tape to retain the gauze dressing and the test substance and to ensure that the animals cannot ingest the test substance Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not recommended The test substance may be wiped from the skin after the six-hour exposure period to prevent ingestion

(9) **Observation period.** The animals should be observed for a period of 21/28 days. Animals in the satellite group, (if one is used) scheduled for follow-up observations should be kept for at least 14 days further without treatment to assess reversibility

(10) **Observation of animals.** (i) Observations should be made at least twice each day for morbidity and mortality Appropriate actions should be taken to minimize loss of animals to the study (e g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals)

(ii) A careful clinical examination should be made at least once prior to the initiation of treatment (to allow for within subject comparisons) and once weekly during treatment in all animals These observations should be made outside the home cage, preferably in a standard arena, and at similar times on each occasion Effort should be made to ensure that variations in the observation conditions are minimal Observations should be detailed and carefully recorded, preferably using scoring systems, explic-

itly defined by the testing laboratory Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e g , lacrimation, piloerection, pupil size, unusual respiratory pattern) Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e g , excessive grooming, repetitive circling) or bizarre behavior (e g , self-mutilation, walking backwards) should be recorded.

(iii) Once, near the end of the exposure period and in any case not earlier than in week 3/4, assessment of motor activity, grip strength, and sensory reactivity to stimuli of different types (e g , visual, auditory, and proprioceptive stimuli) should be conducted Further details of the procedures that could be followed are described in the references listed under paragraphs (h)(1), (h)(3), (h)(4), (h)(5), (h)(6), and (h)(9) of this guideline

(iv) Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits.

(v) Exceptionally, functional observations may be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with functional test performance

(vi) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death

(vii) Food consumption should also be determined weekly if abnormal body weight changes are observed

(viii) Moribund animals should be removed and sacrificed when noticed The time of death should be recorded as precisely as possible.

(ix) At termination, all survivors in the treatment groups should be sacrificed.

(11) **Clinical pathology.** Hematology and clinical chemistry examinations should be made on all animals, including controls, of each sex in each group. The hematology and clinical chemistry parameters should be examined at terminal sacrifice at the end of the study. Overnight fasting of the animals prior to blood sampling is recommended Overall, there is a need for a flexible approach in the measures examined, depending on the observed or expected effects from a chemical, and in the frequency of measures, depending on the duration of potential chemical exposures

(i) Hematology The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet

count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time

(ii) Clinical chemistry (A) Parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity

(B) The recommended clinical chemistry determinations are potassium, sodium, glucose, total cholesterol, urea nitrogen, creatinine, total protein and albumin More than 2 hepatic enzymes, (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, or gamma glutamyl transpeptidase) should also be measured Measurements of additional enzymes (of hepatic or other origin) and bile acids, may also be useful

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include calcium, phosphorus, fasting triglycerides, hormones, methemoglobin, and cholinesterases.

(iii) Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collection appearance, volume, osmolality or specific gravity, pH, protein, glucose, and blood/blood cells

(12) **Ophthalmological examination.** Ophthalmological examinations using an ophthalmoscope or an equivalent device should be made on all animals prior to the administration of the test substance and on all high-dose and control groups at termination If changes in the eyes are detected, all animals in the other dose groups should be examined

(13) **Gross necropsy.** (i) All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents

(ii) The liver, brain, kidneys, spleen, adrenals, testes, epididymides, uterus, ovaries, thymus, and heart should be trimmed and weighed wet, as soon as possible after dissection

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system

(*1*) Salivary glands

(*2*) Esophagus

(*3*) Stomach

(*4*) Duodenum

(*5*) Jejunum

(*6*) Ileum

(*7*) Cecum

(*8*) Colon

(*9*) Rectum.

(*10*) Liver

(*11*) Pancreas

(*12*) Gall bladder (when present)

(B) Nervous system

(*1*) Brain (multiple sections, including cerebrum, cerebellum, and medulla/pons)

(*2*) Pituitary

(*3*) Peripheral nerve(sciatic or tibial, preferably in close proximity to the muscle)

(*4*) Spinal cord (three levels, cervical, mid-thoracic, and lumbar)

(*5*) Eyes (retina, optic nerve)

(C) Glandular system

(*1*) Adrenals

(*2*) Parathyroids

(*3*) Thyroids

(D) Respiratory system

(*1*) Trachea

(*2*) Lung.

(*3*) Pharynx

(*4*) Larynx

(*5*) Nose

(E) Cardiovascular/Hematopoietic system

(*1*) Aorta

(*2*) Heart

(*3*) Bone marrow (and/or fresh aspirate)

(*4*) Lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects)

(*5*) Spleen

(*6*) Thymus

(F) Urogenital system

(*1*) Kidneys

(*2*) Urinary bladder

(*3*) Prostate.

(*4*) Testes.

(*5*) Epididymides

(*6*) Seminal vesicles

(*7*) Uterus

(*8*) Ovaries

(*9*) Female mammary gland

(G) Others

(*1*) All gross lesions and masses

(*2*) Skin (both treated and adjacent untreated areas)

(14) **Histopathology.** (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed under paragraph (e)(13)(iii) of this guideline, of all animals in the control and high-dose groups

(B) All gross lesions in all animals

(C) Target organs in all animals

(D) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing toxic effects in the treated groups

(ii) If excessive early deaths or other problems occur in the high-dose group, compromising the significance of the data, the next dose level should be examined for complete histopathology

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designed for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group—number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion

(ii) When applicable, all observed results, quantitative and qualitative, should be evaluated by an appropriate statistical method Any generally accepted statistical method should be used, the statistical methods including significance criteria should be selected during the design of the study

(2) **Evaluation of study results.** The findings of a 21/28 day dermal toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of toxic effects, and the necropsy and histopathological findings The evaluation should include the relationship between the dose of the test substance, the incidence and severity of abnormalities including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effect on mortality and any other general or specific toxic effects A properly conducted 21/28 day dermal toxicity study should provide information on the effects of repeated application of a substance and a satisfactory estimation of a NOEL. It also can indicate the need for an additional longer-term study and provide information on the selection of dose levels

(3) **Test report.** In addition to reporting requirements specified under EPA Good Laboratory Practice Standards at 40 CFR part 792, subpart J and 40 CFR part 160, and the OECD principles of GLP (ISBN 92–64–12367–9), the following specific information should be reported

(i) Test substance characterization should include

(A) Chemical identification

(B) Lot or batch numbers

(C) Physical properties

(D) Purity/impurities

(E) Identification and composition of any vehicle if used

(ii) Test system should contain data on

(A) Species and strain of animals used and rationale for selection if other than that recommended

(B) Age including body weight data and sex

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(D) Identification of animal diet

(E) Acclimation period

(iii) Test procedure should include the following data:

(A) Method of randomization used

(B) Full description of experimental design and procedure

(C) Dose regime including levels, method, and volume

(iv) Test results should include

(A) Group animal data Tabulation of toxic response data by species, strain, sex, and exposure level for

(*1*) Number of animals exposed

(*2*) Number of animals showing signs of toxicity

(*3*) Number of animals dying

(B) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(*1*) Date of death during the study or whether animals survived to termination

(*2*) Date of observation of each abnormal sign and its subsequent course

(*3*) Body weight data

(*4*) Feed consumption data, when collected

(*5*) Results of ophthalmological examination

(*6*) Results of the hematology tests performed

(*7*) Results of the clinical chemistry tests performed

(*8*) Results of urinalysis, when performed

(*9*) Results of the observations made

(*10*) Necropsy findings, including absolute and relative organ weight data

(*11*) Detailed description of all histopathological findings.

(*12*) Statistical treatment of results, where appropriate

(g) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment The study must be conducted in compliance with GLP regulations

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Crofton K M , Howard J L , Moser V C , Gill M W , Leiter L W , Tilson H A , MacPhail, R C Interlaboratory Comparison of Motor Activity Experiments Implication for Neurotoxicological Assessments *Neurotoxicol Teratol* 13, 599–609 (1991)

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(6) Moser V C , McDaniel K M , Phillips P M Rat Strain and Stock Comparisons using a Functional Observational Battery Baseline Values and Effects of Amitraz *Toxicology and Applied Pharmacology* 108, 267–283 (1991)

(7) National Academy of Sciences Principles and Procedures for Evaluating the Toxicity of Household Substances A report prepared by the Committee for the Revision of NAS Publication 1138, under the aus-

pices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977)

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United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

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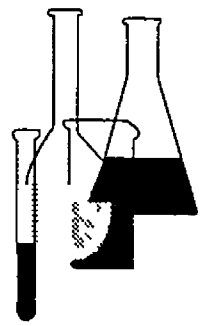
Health Effects Test Guidelines

OPPTS 870.3250
90-Day Dermal Toxicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870 3250 90-Day dermal toxicity

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 798 2250 Dermal Toxicity, OPP 82–3 90–Day Dermal (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 411 Subchronic Dermal Toxicity 90–Day

(b) **Purpose.** In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing The subchronic dermal study has been designed to permit the determination of the no-observed-effect level (NOEL) and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days This study is not capable of determining those effects that have a long latency period for development (e g , carcinogenicity and life shortening) Extrapolation from the results of this study to humans is valid only to a limited degree It can, however, provide useful information on the degree of percutaneous absorption, target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure

(c) **Definitions.** The definitions in section 3 of the Toxic Substance Control Act (TSCA) and the definitions in 40 CFR Part 792--Good Laboratory Practice Standards apply to this test guideline The following definitions also apply to this test guideline

Cumulative toxicity is the adverse effect of repeated doses occurring as a result of prolonged action or increased concentration of the administered test substance or its metabolites in susceptible tissues

Dose in a subchronic dermal study is the amount of test substance applied daily to the skin for 90 days Dose is expressed as weight of the test substance (grams, milligrams), per unit body weight of test animal (milligrams per kilogram), or as weight of the test substance per unit of surface area (milligrams per square centimeter) per day

No-observed-effect level (NOEL) is the maximum dose used in a study which produces no adverse effects The NOEL is expressed in terms of the weight of a test substance given daily per unit weight of test animal (milligrams per kilogram per day)

Subchronic dermal toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the dermal route for a part of the test animal's life span

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this guideline, produces no observable toxic effects or if toxic effects would not be expected based upon data on structurally related compounds, a full study using three dose levels might not be necessary

(e) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** A mammalian species should be used for testing The rat, rabbit, or guinea pig may be used Commonly used laboratory strains should be employed If other mammalian species are used, the tester should provide justification/reasoning for their selection When a subchronic dermal study is conducted as a preliminary to a chronic dermal study, the same species and strain should be used in both studies

(ii) **Age/weight.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization

(B) Dosing should generally begin in guinea pigs between 5–6 weeks of age, in rats between 8–9 weeks of age, and in rabbits at least 12 weeks old

(C) At the commencement of the study, the weight variation of animals used should be within 20 percent of the mean weight for each sex.

(iii) **Sex.** Equal numbers of animals of each sex with healthy skin should be used at each dose level The females should be nulliparous and nonpregnant except for specially designed studies

(iv) **Numbers.** (A) At least 20 animals (10 animals per sex) should be used at each dose level

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before completion of the study

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required

(D) Each animal should be assigned a unique identification number. Dead animals, their preserved organs and tissues, and microscopic slides should be identified by reference to the animal's unique number

(v) **Husbandry.** (A) Animals should be housed in individual cages.

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C

(C) The relative humidity of the experimental animal rooms should be 50 ± 20 percent

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark

(E) Control and test animals should be fed from the same batch and lot The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least five days is recommended

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle If a vehicle or diluent is needed, the vehicle should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance. It is recommended that, whenever possible, the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then solution of other vehicles

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound and if technically feasible, the name and quantities of unknown contaminants and impurities

(iii) If the test substance is dissolved or suspended in a vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(3) **Control groups.** A concurrent control group is required This group should be an untreated or sham-treated control group or, if a vehicle is used in the application of the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or not available, both untreated/sham-treated and vehicle control groups are required

(4) **Satellite group.** A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days In addition a control group of 20 animals (10 animals per sex) should be added to the satellite study

(5) **Dose levels and dose selection.** (i) In subchronic toxicity tests, it is desirable to determine a dose-response relationship as well as a NOEL Therefore, at least three dose levels plus a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest dose level) group should be used Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve.

(ii) The highest dose level should elicit signs of toxicity but not produce severe skin irritation or an incidence of fatality which would prevent a meaningful evaluation If application of the test substance produces severe skin irritation, the concentration may be reduced, although this may result in a reduction in, or absence of, other toxic effects at the high dose level. If the skin has been badly damaged early in the study, it may be necessary to terminate the study and undertake a new one at lower concentrations

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects

(iv) The lowest dose level should not produce any evidence of toxic effects.

(6) **Preparation of animal skin.** Shortly before testing, fur should be clipped from not less than 10 percent of the body surface area for application of the test substance In order to dose approximately 10 percent of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half way down the flank on each side of the animal should be shaved Shaving should be carried out approximately 24 hours before dosing Repeated clipping or shaving is usually needed at approximately weekly intervals When clipping or shaving the fur, care should be taken to avoid abrading the skin which could alter its permeability

(7) **Preparation of test substance.** (i) Liquid test substances are generally used undiluted, except as indicated in paragraph (e)(5)(ii) of this guideline

(ii) Solids should be pulverized when possible The substance should be moistened sufficiently with water or, when necessary, a suitable vehicle to ensure good contact with the skin When a vehicle is used, the influence

of the vehicle on toxicity of, and penetration of the skin by, the test substance should be taken into account

(iii) The volume of application should be kept constant, e g , less than 300 μL for the rat, different concentrations of test solution should be prepared for different dose levels

(8) **Administration of test substance.** (i) The duration of exposure should be at least for 90 days

(ii) Ideally, the animals should be treated with test substance for at least 6 h/day on a 7-day per week basis However, based on practical considerations, application on a 5-day per week basis is acceptable Dosing should be conducted at approximately the same time each day

(iii) The test substance should be applied uniformly over the treatment site.

(iv) The surface area covered may be less for highly toxic substances As much of the area should be covered with as thin and uniform a film as possible

(v) During the exposure period, the test substance should be held in contact with the skin with a porous gauze dressing (less than or equal to 8 ply) The test site should be further covered with nonirritating tape to retain the gauze dressing and the test substance and to ensure that the animals cannot ingest the test substance Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not recommended The test substance may be wiped from the skin after the six-hour exposure period to prevent ingestion

(9) **Observation of animals.** (i) Observations should be made at least twice each day for morbidity and mortality Appropriate actions should be taken to minimize loss of animals to the study (e g , necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals) General clinical observations should be made at least once a day, preferably at the same time each day, taking into consideration the peak period of anticipated effects after dosing The clinical condition of the animal should be recorded

(ii) A careful clinical examination should be made at least once prior to the initiation of treatment (to allow for within subject comparisons) and once weekly during treatment in all animals These observations should be made outside the home cage, preferably in a standard arena, and at similar times on each occasion Effort should be made to ensure that variations in the observation conditions are minimal Observations should be detailed and carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence

of secretions and excretions and autonomic activity (e g , lacrimation, piloerection. pupil size, unusual respiratory pattern) Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e g , excessive grooming, repetitive circling) or bizarre behavior (e g., self-mutilation, walking backwards) should be recorded

(iii) Once, near the end of the exposure period and in any case not earlier than in week 11, assessment of motor activity, grip strength, and sensory reactivity to stimuli of different types (e g , visual, auditory, and proprioceptive stimuli) should be conducted Further details of the procedures that could be followed are described in the references listed under paragraphs (h)(1), (h)(3), (h)(4), (h)(5), (h)(6), and (h)(9) of this guideline

(iv) Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits

(v) Exceptionally, functional observations may be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with functional test performance

(vi) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death.

(vii) Food consumption should also be determined weekly if abnormal body weight changes are observed

(viii) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible

(ix) At termination, all survivors in the control and treatment groups should be sacrificed

(10) **Clinical pathology.** Hematology and clinical chemistry examinations should be made on all animals, including controls, of each sex in each group The hematology and clinical chemistry parameters should be examined at terminal sacrifice at the end of the study Overnight fasting of the animals prior to blood sampling is recommended. Overall, there is a need for a flexible approach in the measures examined, depending on the observed or expected effects from a chemical, and in the frequency of measures, depending on the duration of potential chemical exposures

(i) Hematology The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time

(ii) Clinical chemistry (A) Parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity

(B) The recommended clinical chemistry determinations are potassium, sodium, glucose, total cholesterol, urea nitrogen, creatinine, total protein and albumin More than 2 hepatic enzymes, (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, or gamma glutamyl transpeptidase) should also be measured Measurements of additional enzymes (of hepatic or other origin) and bile acids, may also be useful

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated.

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include calcium, phosphorus, fasting triglycerides, hormones, methemoglobin, and cholinesterases

(iii) Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collection appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells

(11) **Ophthalmological examination.** Using an ophthalmoscope or an equivalent device, ophthalmological examinations should be made on all animals prior to the administration of the test substance and on all high dose and control groups at termination If changes in the eyes are detected, all animals in the other dose groups should be examined.

(12) **Gross necropsy.** (i) All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents

(ii) The liver, brain, kidneys, spleen, adrenals, testes, epididymides, uterus, ovaries, thymus and heart should be trimmed and weighed wet, as soon as possible after dissection

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder (when present)

(B) Nervous system—brain (multiple sections, including cerebrum, cerebellum and medulla/pons) pituitary, peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle), spinal cord (three levels, cervical, mid-thoracic and lumbar), eyes (retina, optic nerve)

(C) Glandular system—adrenals, parathyroid, thyroid

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose

(E) Cardiovascular/Hematopoietic system—aorta, heart, bone marrow (and/or fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen, thymus

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland

(G) Other—all gross lesions and masses, skin (both treated and adjacent untreated areas)

(13) **Histopathology.** (i) The following histopathology should be performed

(A) Full histopathology on the organs and tissues, listed under paragraph (e)(12)(iii) of this guideline, of all animals in the control and high dose groups and all animals that died or were killed during the study

(B) All gross lesions in all animals

(C) Target organs in all animals

(D) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing toxic effects in the treated groups

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group, number of animals at the start of the test, the number of animals showing lesions,

the types of lesions and the percentage of animals displaying each type of lesion

(ii) When applicable, all observed results, qualitative and quantitative, should be evaluated by an appropriate and generally acceptable statistical method Any, generally accepted statistical method should be used, the statistical methods including significance criteria should be selected during the design of the study.

(2) **Evaluation of study results.** The findings of a subchronic dermal toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of toxic effects and the necropsy and histopathological findings The evaluation should include the relationship between the dose of the test substance, the incidence and severity of abnormalities including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effect on mortality, and any other general or specific toxic effects A properly conducted 90-day subchronic dermal study should provide information on the effects of repeated application of a substance and a satisfactory estimation of a NOEL It also can indicate the need for an additional longer-term study and provide information on the selection of dose levels.

(3) **Test report.** In addition to reporting requirements specified under EPA Good Laboratory Practice Standards at 40 CFR part 792, subpart J and 40 CFR part 160, and the OECD principles of GLP (ISBN 92-64-12367-9), the following specific information should be reported·

(i) Test substance characterization should include

(A) Chemical identification

(B) Lot or batch numbers.

(C) Physical properties

(D) Purity/impurities

(ii) Identification and composition of any vehicle if used

(iii) Test system should contain data on

(A) Species and strain of animals used and rationale for selection if other than that recommended

(B) Age including body weight data and sex

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(D) Identification of animal diet

(E) Acclimation period

(iv) Test procedure should include the following data

(A) Method of randomization used

(B) Full description of experimental design and procedure

(C) Dose regime including levels, method, and volume

(v) Test results should include

(A) Group animal data. Tabulation of toxic response data by species, strain, sex and exposure level for

(*1*) Number of animals exposed

(*2*) Number of animals showing signs of toxicity

(*3*) Number of animals dying

(B) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(*1*) Date of death during the study or whether animals survived to termination

(*2*) Date of observation of each abnormal sign and its subsequent course

(*3*) Body weight data

(*4*) Feed consumption data, when collected

(*5*) Results of ophthalmological examination

(*6*) Results of hematological tests performed

(*7*) Results of clinical chemistry tests performed

(*8*) Results of urinalysis, when performed

(*9*) Results of observations made

(*10*) Necropsy findings, including absolute and relative (to body weight) organ weight data

(*11*) Detailed description of all histopathological findings

(*12*) Statistical treatment of results, where appropriate.

(g) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment The study must be conducted in compliance with GLP regulations

(h) **References.** The following references should be consulted for background information on this test guideline

(1) Crofton K M , Howard J L , Moser V C , Gill M W , Leiter L W , Tilson H A , MacPhail, R C Interlaboratory Comparison of Motor Activity Experiments Implication for Neurotoxicological Assessments *Neurotoxicol Teratol* 13, 599-609 (1991)

(2) Draize, J H Dermal toxicity Appraisal of Chemicals in Food, Drugs and Cosmetics The Association of Food and Drug Officials of the United States (1959) 3rd printing 1975 pp 46-59

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(4) International Programme on Chemical Safety Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals Environmental Health Criteria Document No 60 (1986)

(5) Meyer O A , Tilson H A , Byrd W C , Riley M.T A Method for the Routine Assessment of Fore- and Hind-Limb Grip Strength of Rats and Mice *Neurobehav Toxicol* 1, 233-236 (1979)

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(9) Tupper, D E , Wallace R B Utility of the Neurologic Examination in Rats. Acta *Neurobiol Exp* 40, 999-1003 (1980)

(10) United States Environmental Protection Agency Office of Testing and Evaluation Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules 40 CFR Part 772 Standard for Development of Test Data Subpart D FEDERAL REGISTER Vol. 44, No 91. Pp 27350-27362.

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(12) World Health Organization Part I Environmental Health Criteria 6, Principles and Methods for Evaluating the Toxicity of Chemicals (Geneva World Health Organization, 1978)

(13) World Health Organization Guidelines for Evaluation of Drugs for Use in Man, WHO Technical Report Series No 563 (Geneva World Health Organization, 1975)

(14) World Health Organization Principles for Pre-Clinical Testing of Drug Safety, WHO Technical Report Series No 341 (Geneva. WHO, 1966)

United States
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Prevention Pesticides
and Toxic Substances
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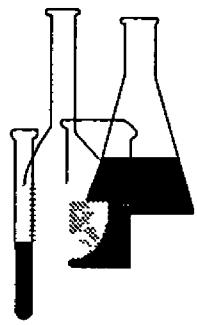
Health Effects Test Guidelines OPPTS 870.3465 90-Day Inhalation Toxicity



INTRODUCTION

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The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in title 40, chapter I, subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S.C 136, *et seq*)

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OPPTS 870.3465 90-Day inhalation toxicity.

(a) **Scope—(1) Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 798 2450 Inhalation Toxicity, OPP 82-4 90-Day Inhalation—Rat (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09-82-025, 1982, and OECD 413 Subchronic Inhalation Toxicity· 90-Day

(b) **Purpose.** In the assessment and evaluation of the toxic characteristics of a gas, volatile substance, or aerosol/particulate, determination of subchronic inhalation toxicity may be carried out after initial information on toxicity has been obtained by acute testing The subchronic inhalation study has been designed to permit the determination of the no-observed-effect-level (NOEL) and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days This study is not capable of determining those effects that have a long latency period for development (e g , carcinogenicity and life shortening) Extrapolation from the results of this study to humans is valid only to a limited degree. It can, however, provide useful information on health hazards likely to arise from repeated exposures by the inhalation route over a limited period of time It will provide information on target organs and the possibilities of accumulation, and can be of use in selecting concentration levels for chronic studies and establishing safety criteria for human exposure Hazards of inhaled substances are influenced by the inherent toxicity and by physical factors such as volatility and particle size.

(c) **Definitions.** The definitions in section 3 of the Toxic Substance Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline

Aerodynamic equivalent diameter is defined as the diameter of a unit density sphere having the same terminal settling velocity as the particle in question, whatever its size, shape, and density It is used to predict where in the respiratory tract such particles may be deposited

Concentration in a subchronic inhalation study is the amount of test substance administered via inhalation for a period of 90 days Concentration is expressed as weight of the test substance per unit volume of air (milligrams per liter or parts per million)

Cumulative toxicity is the adverse effects of repeated concentration occurring as a result of prolonged action on, or increased concentration of the administered test substance or its metabolites in susceptible tissues

Inhalable diameter refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract

No-observed-effect-level (NOEL) is the maximum concentration used in a study which produces no adverse effects

Mass median aerodynamic diameter (MMAD) is the median aerodynamic diameter and along with the geometric standard deviation (GSD) is used to describe the particle size distribution of any aerosol statistically based on the weight and size of the particles Fifty percent of the particles by weight will be smaller than the median diameter and 50 percent of the particles will be larger

Subchronic inhalation toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by inhalation for part (approximately 10 percent) of a life span

(d) **Limit test.** If exposure at a concentration of 1 mg/L (expected human exposure may indicate the need for a higher concentration), or where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration produces no observable toxic effects, then a full study using three concentrations might not be necessary

(e) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** A mammalian species shall be used for testing A variety of rodent species may be used, although the rat is the preferred species Commonly used laboratory strains should be employed If another mammalian species is used, the tester shall provide justification/reasoning for its selection

(ii) **Age/weight.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization.

(B) Dosing of rodents should generally begin no later than 8–9 weeks of age

(C) At the commencement of the study the weight variation of animals used shall not exceed ± 20 percent of the mean weight for each sex

(iii) **Sex.** (A) Equal numbers of animals of each sex shall be used at each concentration

(B) Females shall be nulliparous and nonpregnant

(iv) **Numbers.** (A) At least 20 animals (10 females and 10 males) should be used for each test group

(B) If interim sacrifices are planned, the number of animals shall be increased by the number of animals scheduled to be sacrificed before the completion of the study

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required

(D) Each animal shall be assigned a unique identification number Dead animals, their preserved organs and tissues, and microscopic slides shall be identified by reference to the animal's unique number

(v) **Husbandry.** (A) Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g , morbidity, excitability) may indicate a need for individual caging Animals must be housed individually in inhalation chambers during exposure to aerosols

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C

(C) The relative humidity of the experimental animal rooms should be 30–70 percent

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark

(E) Control and test animals should be fed from the same batch and lot. The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least 5 days is recommended

(2) **Control and test substances.** (i) Whenever it is necessary to formulate the test substance with a vehicle for aerosol generation, the vehicle ideally should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test substance and, if technically feasible, the name and quantities of unknown contaminants and impurities

(3) **Control groups.** A concurrent control group is required This group shall be an untreated or sham-treated control group Except for treatment with the test substance, animals in the control group shall be handled in a manner identical to the test group animals Where a vehicle other than water is used to generate a substance, a vehicle control group should be used If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required

(4) **Satellite group.** A satellite group of 20 animals (10 animals per sex) may be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days In addition, a control group of 20 animals (10 animals of each sex) should be added to the satellite study

(5) **Concentration levels and concentration selection.** (i) In subchronic toxicity tests, it is desirable to have a concentration-response relationship as well as a NOEL Therefore, at least three concentration levels plus a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall be used Concentrations should be spaced appropriately to produce test groups with a range of toxic effects The data should be sufficient to produce a concentration-response curve

(ii) The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation

(iii) The intermediate concentrations should be spaced to produce a gradation of toxic effects

(iv) The lowest concentration should produce no evidence of toxicity

(v) In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations

(6) **Administration of the test substance.** Animals should be exposed to the test substance for 6 h per day on a 7-day per week basis for a period of at least 90 days Based primarily on practical considerations, exposure for 6 h per day on a 5-day per week basis is acceptable

(7) **Observation period.** The animals should be observed for a period of 90 days Animals in the satellite group (if used) scheduled for follow-up observations should be kept for at least 28 days further without treatment to assess reversibility

(8) **Exposure specifications.** (i) The animals shall be tested in dynamic inhalation equipment designed to sustain a minimum airflow of 10 air changes per hour, an adequate oxygen content of at least 19 percent, and uniform conditions throughout the exposure chamber Maintenance of

slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas It is not normally necessary to measure chamber oxygen concentration if airflow is adequate

(ii) The selection of a dynamic inhalation chamber should be appropriate for the test substance and test system Where a whole body chamber is used to expose animals to an aerosol, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance To ensure stability of a chamber atmosphere, the total volume occupied by the test animals shall not exceed 5 percent of the volume of the test chamber It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order to minimize oral exposures due to animals licking compound off their fur Heat stress should be minimized

(iii) The temperature at which the test is performed should be maintained at 22 ±2 °C The relative humidity should be maintained between 40–60 percent, but in certain instances (e g , use of water vehicle) this may not be practicable

(9) **Physical measurements.** Measurements or monitoring shall be made of the following

(i) The rate of airflow shall be monitored continuously but recorded at least three times during the exposure

(ii) The actual concentrations of the test substance shall be measured in the animal's breathing zone During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable and monitored continuously or intermittently depending on the method of analysis. Chamber concentration may be measured using gravimetric or analytical methods as appropriate If trial run measurements are reasonably consistent (±10 percent for liquid aerosol, gas, or vapor, ±20 percent for dry aerosol), then two measurements should be sufficient If measurements are not consistent, three to four measurements should be taken Whenever the test substance is a formulation, or it is necessary to formulate the test substance with a vehicle for aerosol generation, the analytical concentration must be reported for the total formulation, and not just for the active ingredient (AI) If, for example, a formulation contains 10 percent AI and 90 percent inerts, a chamber analytical limit concentration of 2 mg/L would consist of 0 2 mg/L of the AI It is not necessary to analyze inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation, the grounds for this conclusion must be provided in the study report If there is some difficulty in measuring chamber analytical concentration due to precipitation, nonhomogeneous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary

(iii) During the development of the generating system, particle size analysis shall be performed to establish the stability of aerosol concentrations with respect to particle size The MMAD particle size range should be between 1–3 μm The particle size of hygroscopic materials should be small enough when dry to assure that the size of the swollen particle will still be within the 1–3 μm range Measurements of aerodynamic particle size in the animal's breathing zone should be measured during a trial run If MMAD values for each exposure level are within 10 percent of each other, then two measurements during the exposures should be sufficient. If pretest measurements are not within 10 percent of each other, three to four measurements should be taken

(iv) Temperature and humidity shall be monitored continuously and recorded at least three times during an exposure

(10) **Feed and water during exposure period.** Feed shall be withheld during exposure Water may also be withheld during exposure

(11) **Observation of animals.** (i) During and following exposure, observations are made and recorded systematically, individual records should be maintained for each animal It is not always possible to observe animals during exposure in a whole-body chamber

(ii) Observations shall be made at least twice each day for morbidity and mortality Appropriate actions should be taken to minimize loss of animals to the study (e g , Necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) General clinical observations should be made at least once a day, preferably at the same time each day, taking into consideration the peak period of anticipated effects after dosing The clinical condition of the animal should be recorded

(iv) A careful clinical examination should be made at least once prior to the initiation of treatment (to allow for within subject comparisons) and once weekly during treatment in all animals These observations should be made outside the home cage, preferably in a standard arena, and at similar times on each occasion Effort should be made to ensure that variations in the observation conditions are minimal Observations should be detailed and carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e g., lacrimation, piloerection, pupil size, unusual respiratory pattern) Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e g , excessive grooming, repetitive circling) or bizarre behavior (e g , self-mutilation, walking backwards) should be recorded.

(v) Once, near the end of the exposure period and in any case not earlier than in week 11 assessment of motor activity, grip strength, and sensory reactivity to stimuli of different types (e g , visual, auditory, and proprioceptive stimuli) should be conducted Further details of the procedures that could be followed are described in the references listed under paragraphs (h)(3), (h)(4), (h)(5), (h)(7), (h)(8), and (h)(11) of this guideline

(vi) Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits

(vii) Exceptionally, functional observations may be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with functional test performance

(viii) Individual weights of animals shall be determined shortly before the test substance is administered, and weekly thereafter

(ix) Food consumption shall also be determined weekly if abnormal body weight changes are observed

(x) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible

(xi) At termination, all survivors in the treatment groups shall be sacrificed

(12) **Clinical pathology.** Hematology and clinical chemistry examinations should be made on all animals, including controls, of each sex in each group The hematology and clinical chemistry parameters should be examined at terminal sacrifice at the end of the study Overnight fasting of the animals prior to blood sampling is recommended Overall, there is a need for a flexible approach in the measures examined, depending on the observed or expected effects from a chemical, and in the frequency of measures, depending on the duration of potential chemical exposures

(i) Hematology The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time

(ii) Clinical chemistry (A) Parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity

(B) The recommended clinical chemistry determinations are potassium, sodium, glucose, total cholesterol, urea nitrogen, creatinine, total protein and albumin More than 2 hepatic enzymes, (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, or gamma glutamyl transpeptidase) should also be measured. Measurements of addtional enzymes (of hepatic or other origin) and bile acids, may also be useful

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include calcium, phosphorus, fasting triglycerides, hormones, methemoglobin, and cholinesterases

(iii) Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collection· appearance, volume, osmolality or specific gravity, pH, protein, glucose, and blood/blood cells

(13) **Ophthalmological examination.** Ophthalmological examinations using an ophthalmoscope or an equivalent device should be made on all animals prior to the administration of the test substance and on all high dose and control groups at termination If changes in the eyes are detected, all animals in the other dose groups should be examined

(14) **Gross necropsy.** (i) All animals shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices and the cranial, thoracic, and abdominal cavities and their contents.

(ii) The liver, kidneys, adrenals, testes, epididymides, ovaries, uterus, thymus, spleen, brain, and heart shall be trimmed and weighed wet, as soon as possible after dissection to avoid drying

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system

(*1*) Salivary glands

(*2*) Esophagus

(*3*) Stomach

(*4*) Duodenum

(*5*) Jejunum.

(*6*) Ileum

(*7*) Cecum

(*8*) Colon

(*9*) Rectum

(*10*) Liver

(*11*) Pancreas

(*12*) Gallbladder (where present)

(B) Nervous system

(*1*) Brain (including sections of medulla/pons, cerebellum, and cerebrum).

(*2*) Pituitary

(*3*) Peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle)

(*4*) Spinal cord (three levels cervical, mid-thoracic, and lumbar).

(*5*) Eyes (retina, optic nerve)

(C) Glandular system

(*1*) Adrenals

(*2*) Parathyroids

(*3*) Thyroids

(D) Respiratory system

(*1*) Trachea

(*2*) Lung

(*3*) Pharynx

(*4*) Larynx

(*5*) Nose

(E) Cardiovascular/hematopoietic system

(*1*) Aorta

(*2*) Heart

(*3*) Bone marrow (and/or fresh aspirate)

(*4*) Lymph nodes (preferably one node covering the route of administration and another one distant from the route of administration)

(*5*) Spleen

(*6*) Thymus

(F) Urogenital system

(*1*) Kidneys

(*2*) Urinary bladder

(*3*) Prostate

(*4*) Testes

(*5*) Epididymides

(*6*) Seminal vesicle(s)

(*7*) Uterus

(*8*) Ovaries

(*9*) Female mammary gland

(G) Other

(*1*) Lacrimal gland

(*2*) Skin

(*3*) All gross lesions and masses

(15) **Histopathology.** (i) The following histopathology shall be performed:

(A) Full histopathology on the respiratory tract and other organs and tissues, listed under paragraph (e)(15)(iii) of this guideline, of all animals in the control and high exposure groups and all animals that died or were killed during the study

(B) All gross lesions in all animals

(C) Target organs in all animals

(D) Lungs of all animals Special attention to examination of the respiratory tract should be made for evidence of infection as this provides a convenient assessment of the state of health of the animals

(E) When a satellite group is used, histopathology shall be performed on tissues and organs identified as showing effects in the treated groups

(ii) If excessive early deaths or other problems occur in the high exposure group compromising the significance of the data, the next concentration should be examined for complete histopathology

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion

(ii) When applicable, all observed results, quantitative and qualitative, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used, the statistical methods including significance criteria should be selected during the design of the study

(2) **Evaluation of study results.** The findings of the subchronic inhalation toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings The evaluation will include the relationship between the concentration of the test substance and duration of exposure, and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level. It also can indicate the need for an additional longer-term study and provide information on the selection of concentrations

(3) **Test report.** In addition to reporting requirements specified under EPA Good Laboratory Practice Standards, 40 CFR part 792, subpart J and 40 CFR part 160, and the OECD principles of GLP (ISBN 92–64–12367–9) the following specific information shall be reported

(i) Test substance characterization shall include

(A) Chemical identification

(B) Lot or batch number

(C) Physical properties

(D) Purity/impurities

(E) Identification and composition of any vehicle used

(ii) Test system information shall include

(A) Species and strain of animals used and rationale for selection if other than that recommended

(B) Age, sex, and body weight data

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(D) Identification of animal diet

(E) Acclimation period

(iii) Test procedure information shall include

(A) Method of randomization used

(B) Full description of experimental design and procedure

(C) Exposure regimen including levels, methods, and volume

(D) Test conditions The following exposure conditions must be reported·

(*1*) Description of exposure apparatus including design, type, volume, source of air, system for generating aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described

(E) Exposure data These shall be tabulated and presented with mean values and a measure of variability (e g , standard deviation) and should include

(*1*) Airflow rates through the inhalation equipment

(*2*) Temperature and humidity of air

(*3*) Actual (analytical or gravimetric) concentration in the breathing zone.

(*4*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air)

(*5*) Particle size distribution, calculated mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD)

(*6*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable) and the efforts taken to comply with this aspect of the guidelines

(iv) Test results (A) Group animal data Tabulation of toxic response data by species, strain, sex, and exposure level for

(*1*) Number of animals exposed

(*2*) Number of animals showing signs of toxicity

(*3*) Number of animals dying

(B) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(*1*) Time of death during the study or whether animals survived to termination

(*2*) Time of observation of each abnormal sign and its subsequent course.

(*3*) Body weight data

(*4*) Feed consumption data, when collected

(*5*) Results of ophthalmological examination

(*6*) Results of hematological tests performed

(*7*) Results of clinical chemistry tests performed

(*8*) Results of urinalysis, if performed

(*9*) Results of observations made

(*10*) Necropsy findings, including absolute and relative organ weight data.

(*11*) Detailed description of all histopathological findings.

(*12*) Statistical treatment of results, where appropriate

(g) **Quality control.** A system shall be developed and maintained to assure and document adequate performance of laboratory staff and equipment The study must be conducted in compliance with GLP regulations

(h) **References.** The following references should be consulted for additional background information on this test guideline

(1) Cage, J C Experimental Inhalation Toxicology, Methods in Toxicology Ed G E Paget (Philadelphia F A Davis Co , 1970) pp 258–277

(2) Casarett, L J and Doull J Chapter 9, Toxicology The Basic Science of Poisons (New York Macmillan Publishing Co Inc , 1975).

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(4) Gad S C A Neuromuscular Screen for Use in Industrial Toxicology *Journal of Toxicology and Environmental Health* 9, 691–704 (1982)

(5) International Programme on Chemical Safety Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals Environmental Health Criteria Document No 60 (1986)

(6) MacFarland, H N Respiratory Toxicology, Essays in Toxicology Ed W.J Hayes Vol 7 (New York Academic Press, 1976) pp. 121–154.

(7) Meyer O A , Tilson H A , Byrd W C , Riley M T. A Method for the Routine Assessment of Fore- and Hind-Limb Grip Strength of Rats and Mice *Neurobehav Toxicol* 1, 233–236 (1979)

(8) Moser V C , McDaniel K M , Phillips P.M Rat Strain and Stock Comparisons using a Functional Observational Battery. Baseline Values and Effects of Amitraz *Toxicol Appl Pharmacol* 108, 267–283 (1991)

(9) National Academy of Sciences Principles and Procedures for Evaluating the Toxicity of Household Substances, a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977)

(10) Organization for Economic Cooperation and Development Guidelines for testing of chemicals, Section 4—Health Effects, Part—413 Subchronic Inhalation Toxicity Studies (Paris, 1981)

(11) Tupper, D E , Wallace R B Utility of the Neurologic Examination in Rats Acta. *Neurobiol Exp* 40, 999–1003 (1980)

(12) U S Environmental Protection Agency, Office of Testing and Evaluation. Proposed health effects test standards for toxic substances control act test rules, 40 CFR Part 772 Standard for Development of Test Data Subpart D FEDERAL REGISTER (44 FR 27350–27362)

(13) U S Environmental Protection Agency, Office of Pesticide Programs, Health Effects Division Interim policy for particle size and limit concentration issues in inhalation toxicity studies (February 1, 1994)

(14) Weingand K, Brown G, Hall R et al Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies *Fundam & Appl Toxicol* 29 198–201 (1996)

(15) World Health Organization Part I Environmental Health Criteria 6, Principles and Methods for Evaluating the Toxicity of Chemicals (Geneva World Health Organization, 1978)

(16) World Health Organization Principles for pre-clinical testing of drug safety *WHO Technical Report Series No 341* (Geneva World Health Organization, 1966)

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Prevention Pesticides
and Toxic Substances
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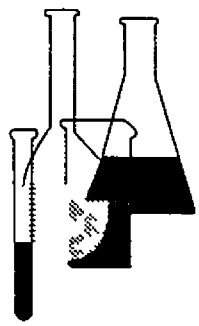

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

Final Guideline Release: This guideline is available from the U S Government Printing Office, Washington, DC 20402 on disks or paper copies call (202) 512–0132 This guideline is also available electronically in ASCII and PDF (portable document format) from EPA's World Wide Web site (http //www epa gov/epahome/research htm) under the heading ''Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines ''

OPPTS 870 3700 Prenatal developmental toxicity study

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*), as amended by the Food Quality Protection Act (FQPA)(Pub L 104–170), and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is the OPPT guideline under 40 CFR 798 4900, OPP guideline 83–3, and OECD guideline 414

(b) **Purpose.** This guideline for developmental toxicity testing is designed to provide general information concerning the effects of exposure of the pregnant test animal on the developing organism, this may include death, structural abnormalities, or altered growth and an assessment of maternal effects For information on testing for functional deficiencies and other postnatal effects, the guidelines for the two-generation reproductive toxicity study and the developmental neurotoxicity study should be consulted

(c) **Good laboratory practice standards.** The study should be conducted in accordance with the laboratory practices stipulated in 40 CFR Part 160 (FIFRA) and 40 CFR Part 792 (TSCA)—Good Laboratory Practice Standards.

(d) **Principle of the test method.** The test substance is administered to pregnant animals at least from implantation to one day prior to the expected day of parturition Shortly before the expected date of delivery, the pregnant females are terminated, the uterine contents are examined, and the fetuses are processed for visceral and skeletal evaluation

(e) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** It is recommended that testing be performed in the most relevant species, and that laboratory species and strains which are commonly used in prenatal developmental toxicity testing be employed The preferred rodent species is the rat and the preferred non-rodent species is the rabbit

(ii) **Age.** Young adult animals should be used

(iii) **Sex.** Nulliparous female animals should be used at each dose level Animals should be mated with males of the same species and strain, avoiding the mating of siblings, if parentage is known Day 0 in the test is the day on which a vaginal plug and/or sperm are observed in the rodent or that insemination is performed or observed in the rabbit

(iv) **Animal care.** Animal care and housing should be in accordance with the recommendations contained in the DHHS/PHS NIH Publication No 86–23, 1985, *Guidelines for the Care and Housing of Laboratory Animals*, or other appropriate guidelines

(v) **Number of animals.** Each test and control group should contain a sufficient number of animals to yield approximately 20 animals with implantation sites at necropsy

(2) **Administration of test and control substances—(i) Dose levels and dose selection.** (A) At least three-dose levels and a concurrent control should be used Healthy animals should be randomly assigned to the control and treatment groups, in a manner which results in comparable mean body weight values among all groups The dose levels should be spaced to produce a gradation of toxic effects Unless limited by the physical/chemical nature or biological properties of the test substance, the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity but not death or severe suffering. In the case of maternal mortality, this should not be more than approximately 10 percent The intermediate dose levels should produce minimal observable toxic effects. The lowest dose level should not produce any evidence of either maternal or developmental toxicity (i e , the no-observed-adverse-effect level, NOAEL) or should be at or near the limit of detection for the most sensitive endpoint Two- or four-fold intervals are frequently optimal for spacing the dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e g , more than a factor of 10) between dosages

(B) It is desirable that additional information on metabolism and pharmacokinetics of the test substance be available to demonstrate the adequacy of the dosing regimen This information should be available prior to testing

(C) The highest dose tested need not exceed 1,000 mg/kg/day by oral or dermal administration, or 2 mg/L (or the maximum attainable concentration) by inhalation, unless potential human exposure data indicate the need for higher doses If a test performed at the limit dose level, using the procedures described for this study, produces no observable toxicity and if an effect would not be expected based upon data from structurally related compounds, then a full study using three-dose levels may not be considered necessary.

(ii) **Control group.** (A) A concurrent control group should be used This group should be a sham-treated control group or a vehicle-control group if a vehicle is used in administering the test substance

(B) The vehicle control group should receive the vehicle in the highest volume used

(C) If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics Effects on the absorption, distribution, metabolism, or retention of the test substance, effects on the chemical properties of the test substance which may alter its toxic

characteristics, and effects on the food or water consumption or the nutritional status of the animals

(iii) **Route of administration** (A) The test substance or vehicle is usually administered orally by intubation

(B) If another route of administration is used, for example, when the route of administration is based upon the principal route of potential human exposure, the tester should provide justification and reasoning for its selection, and appropriate modifications may be necessary Further information on dermal or inhalation exposure is provided under paragraphs (h)(12), (h)(28), and (h)(29) of this guideline Care should be taken to minimize stress on the maternal animals For materials administered by inhalation, whole-body exposure is preferable to nose-only exposure due to the stress of restraint required for nose-only exposure

(C) The test substance should be administered at approximately the same time each day

(D) When administered by gavage or dermal application, the dose to each animal should be based on the most recent individual body weight determination

(iv) **Dosing schedule.** At minimum, the test substance should be administered daily from implantation to the day before cesarean section on the day prior to the expected day of parturition Alternatively, if preliminary studies do not indicate a high potential for preimplantation loss, treatment may be extended to include the entire period of gestation, from fertilization to approximately 1 day prior to the expected day of termination

(f) **Observation of animals—(1) Maternal.** (i) Each animal should be observed at least once daily, considering the peak period of anticipated effects after dosing. Mortality, moribundity, pertinent behavioral changes, and all signs of overt toxicity should be recorded at this cageside observation. In addition, thorough physical examinations should be conducted at the same time maternal body weights are recorded.

(ii) Animals should be weighed on day 0, at termination, and at least at 3–day intervals during the dosing period

(iii) Food consumption should be recorded on at least 3-day intervals, preferably on days when body weights are recorded

(iv) Termination schedule (A) Females should be terminated immediately prior to the expected day of delivery

(B) Females showing signs of abortion or premature delivery prior to scheduled termination should be killed and subjected to a thorough macroscopic examination

(v) Gross necropsy At the time of termination or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy Evaluation of the dams during cesarean section and subsequent fetal analyses should be conducted without knowledge of treatment group in order to minimize bias

(vi) Examination of uterine contents (A) Immediately after termination or as soon as possible after death, the uteri should be removed and the pregnancy status of the animals ascertained Uteri that appear nongravid should be further examined (e g by ammonium sulfide staining) to confirm the nonpregnant status

(B) Each gravid uterus (with cervix) should be weighed Gravid uterine weights should not be obtained from dead animals if autolysis or decomposition has occurred

(C) The number of corpora lutea should be determined for pregnant animals

(D) The uterine contents should be examined for embryonic or fetal deaths and the number of viable fetuses The degree of resorption should be described in order to help estimate the relative time of death of the conceptus

(2) **Fetal.** (i) The sex and body weight of each fetus should be determined

(ii) Each fetus should be examined for external anomalies.

(iii) Fetuses should be examined for skeletal and soft tissue anomalies (e g variations and malformations or other categories of anomalies as defined by the performing laboratory)

(A) For rodents, approximately one-half of each litter should be prepared by standard techniques and examined for skeletal alterations, preferably bone and cartilage The remainder should be prepared and examined for soft tissue anomalies, using appropriate serial sectioning or gross dissection techniques It is also acceptable to examine all fetuses by careful dissection for soft tissue anomalies followed by an examination for skeletal anomalies

(B) For rabbits, all fetuses should be examined for both soft tissue and skeletal alterations The bodies of these fetuses should be evaluated by careful dissection for soft-tissue anomalies, followed by preparation and examination for skeletal anomalies An adequate evaluation of the internal structures of the head, including the eyes, brain, nasal passages, and tongue, should be conducted for at least half of the fetuses

(g) **Data and reporting**—(1) **Treatment of results.** Data should be reported individually and summarized in tabular form, showing for each test group the types of change and the number of dams, fetuses, and litters displaying each type of change

(2) **Evaluation of study results.** The following should be provided

(i) Maternal and fetal test results, including an evaluation of the relationship, or lack thereof, between the exposure of the animals to the test substance and the incidence and severity of all findings

(ii) Criteria used for categorizing fetal external, soft tissue, and skeletal anomalies

(iii) When appropriate, historical control data to enhance interpretation of study results Historical data (on litter incidence and fetal incidence within litter), when used, should be compiled, presented, and analyzed in an appropriate and relevant manner In order to justify its use as an analytical tool, information such as the dates of study conduct, the strain and source of the animals, and the vehicle and route of administration should be included

(iv) Statistical analysis of the study findings should include sufficient information on the method of analysis, so that an independent reviewer/ statistician can reevaluate and reconstruct the analysis In the evaluation of study data, the litter should be considered the basic unit of analysis

(v) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered

(3) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, and 40 CFR part 160, subpart J, the following specific information should be reported Both individual and summary data should be presented.

(i) Species and strain

(ii) Maternal toxic response data by dose, including but not limited to

(A) The number of animals at the start of the test, the number of animals surviving, the number pregnant, and the number aborting

(B) Day of death during the study or whether animals survived to termination

(C) Day of observation of each abnormal clinical sign and its subsequent course

(D) Body weight and body weight change data, including body weight change adjusted for gravid uterine weight

(E) Food consumption and, if applicable, water consumption data

(F) Necropsy findings, including gravid uterine weight

(iii) Developmental endpoints by dose for litters with implants, including

(A) Corpora lutea counts

(B) Implantation data, number and percent of live and dead fetuses, and resorptions (early and late)

(C) Pre- and postimplantation loss calculations

(iv) Developmental endpoints by dose for litters with live fetuses, including

(A) Number and percent of live offspring

(B) Sex ratio

(C) Fetal body weight data, preferably by sex and with sexes combined

(D) External, soft tissue, and skeletal malformation and variation data The total number and percent of fetuses and litters with any external, soft tissue, or skeletal alteration, as well as the types and incidences of individual anomalies, should be reported

(v) The numbers used in calculating all percentages or indices.

(vi) Adequate statistical treatment of results

(vii) A copy of the study protocol and any amendments should be included.

(h) **References.** The following references should be consulted for additional background information on this test guideline

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Prevention Pesticides
and Toxic Substances
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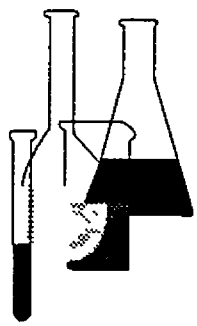
Health Effects Test Guidelines OPPTS 870.3800 Reproduction and Fertility Effects



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies call (202) 512–0132 This guideline is also available electronically in ASCII and PDF (portable document format) from EPA's World Wide Web site (http //www epa gov/epahome/research.htm) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines "

OPPTS 870.3800 Reproduction and fertility effects

(a) **Scope**—(1) **Applicability** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*), as amended by the Food Quality Protection Act (FQPA)(Pub L 104–170) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is the OPPT guideline under 40 CFR 798 4700, OPP guideline 83–4, and OECD guideline 416

(b) **Purpose.** This guideline for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, target organs in the offspring, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests Additionally, since the study design includes *in utero* as well as postnatal exposure, this study provides the opportunity to examine the susceptibility of the immature/neonatal animal For further information on functional deficiencies and developmental effects, additional study segments can be incorporated into the protocol, utilizing the guidelines for developmental toxicity or developmental neurotoxicity

(c) **Good laboratory practice standards.** The study should be conducted in accordance with the laboratory practices stipulated in 40 CFR Part 160 (FIFRA) and 40 CFR Part 792 (TSCA)—Good Laboratory Practice Standards

(d) **Principle of the test method.** The test substance is administered to parental (P) animals prior to and during their mating, during the resultant pregnancies, and through the weaning of their F1 offspring The substance is then administered to selected F1 offspring during their growth into adulthood, mating, and production of an F2 generation, until the F2 generation is weaned

(e) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** The rat is the most commonly used species for testing If another mammalian species is used, the tester should provide justification/reasoning for its selection, and appropriate modifications will be necessary Healthy parental animals, which have been acclimated to laboratory conditions for at least 5 days and have not been subjected to previous experimental procedures, should be used Strains of low fecundity should not be used

(ii) **Age.** Parental (P) animals should be 5 to 9 weeks old at the start of dosing The animals of all test groups should be of uniform weight,

age, and parity as nearly as practicable, and should be representative of the species and strain under study

(iii) **Sex.** (A) For an adequate assessment of fertility, both males and females should be studied

(B) The females should be nulliparous and nonpregnant

(iv) **Animal care.** Animal care and housing should be in accordance with the recommendations contained in DHHS/PHS NIH Publication No 86–23, 1985, *Guidelines for the Care and Use of Laboratory Animals*, or other appropriate guidelines

(v) **Number of animals.** Each control group should contain a sufficient number of mating pairs to yield approximately 20 pregnant females Each test group should contain a similar number of mating pairs

(vi) **Identification of animals.** Each animal should be assigned a unique identification number For the P generation, this should be done before dosing starts For the F1 generation, this should be done for animals selected for mating, in addition, records indicating the litter of origin should be maintained for all selected F1 animals

(2) **Administration of test and control substances—(i) Dose levels and dose selection.** (A) At least three-dose levels and a concurrent control should be used. Healthy animals should be randomly assigned to the control and treatment groups, in a manner which results in comparable mean body weight values among all groups The dose levels should be spaced to produce a gradation of toxic effects Unless limited by the physical/chemical nature or biological properties of the test substance, the highest dose should be chosen with the aim to induce some reproductive and/or systemic toxicity but not death or severe suffering. In the case of parental mortality, this should not be more than approximately 10 percent. The intermediate dose levels should produce minimal observable toxic effects. The lowest dose level should not produce any evidence of either systemic or reproductive toxicity (i e , the no-observed-adverse-effect level, NOAEL) or should be at or near the limit of detection for the most sensitive endpoint Two- or four-fold intervals are frequently optimal for spacing the dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e g , more than a factor of 10) between dosages.

(B) It is desirable that additional information on metabolism and pharmacokinetics of the test substance be available to demonstrate the adequacy of the dosing regimen This information should be available prior to testing

(C) The highest dose tested should not exceed 1,000 mg/kg/day (or 20,000 ppm in the diet), unless potential human exposure data indicate

the need for higher doses If a test performed at the limit dose level, using the procedures described for this study, produces no observable toxicity and if an effect would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary

(ii) **Control group.** (A) A concurrent control group should be used This group should be an untreated or sham treated group or a vehicle-control group if a vehicle is used in administering the test substance

(B) If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used

(C) If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics Effects on the absorption, distribution, metabolism, or retention of the test substance, effects on the chemical properties of the test substance which may alter its toxic characteristics, and effects on the food or water consumption or the nutritional status of the animals

(D) If a test substance is administered in the diet and causes reduced dietary intake or utilization, the use of a pair-fed control group may be considered necessary

(iii) **Route of administration.** (A) The test substance is usually administered by the oral route (diet, drinking water, or gavage).

(B) If administered by gavage or dermal application, the dosage administered to each animal prior to mating and during gestation and lactation should be based on the individual animal body weight and adjusted weekly at a minimum

(C) If another route of administration is used, for example, when the route of administration is based upon the principal route of potential human exposure, the tester should provide justification and reasoning for its selection, and appropriate modifications may be necessary Further information on dermal or inhalation exposure is provided under paragraphs (g)(18) and (g)(19) of this guideline Care should be taken to minimize stress on the maternal animals and their litters during gestation and lactation

(D) All animals should be dosed by the same method during the appropriate experimental period

(iv) **Dosing schedule.** (A) The animals should be dosed with the test substance on a 7–days–a–week basis

(B) Daily dosing of the parental (P) males and females should begin when they are 5 to 9 weeks old Daily dosing of the F1 males and females

should begin at weaning For both sexes (P and F1), dosing should be continued for at least 10 weeks before the mating period

(C) Daily dosing of the P and F1 males and females should continue until termination

(3) **Mating procedure—(i) Parental.** (A) For each mating, each female should be placed with a single randomly selected male from the same dose level (1 1 mating) until evidence of copulation is observed or either 3 estrous periods or 2 weeks has elapsed Animals should be separated as soon as possible after evidence of copulation is observed If mating has not occurred after 2 weeks or 3 estrous periods, the animals should be separated without further opportunity for mating Mating pairs should be clearly identified in the data

(B) Vaginal smears should be collected daily and examined for all females during mating, until evidence of copulation is observed.

(C) Each day, the females should be examined for presence of sperm or vaginal plugs Day 0 of pregnancy is defined as the day a vaginal plug or sperm are found

(ii) **F1 mating.** For mating the F1 offspring, at least one male and one female should be randomly selected from each litter for mating with another pup of the same dose level but different litter, to produce the F2 generation

(iii) **Second mating.** In certain instances, such as poor reproductive performance in the controls, or in the event of treatment-related alterations in litter size, the adults may be remated to produce an F1b or F2b litter If production of a second litter is deemed necessary in either generation, the dams should be remated approximately 1–2 weeks following weaning of the last F1a or F2a litter

(iv) **Special housing.** After evidence of copulation, animals that are presumed to be pregnant should be caged separately in delivery or maternity cages. Pregnant animals should be provided with nesting materials when parturition is near

(v) **Standardization of litter sizes.** (A) Animals should be allowed to litter normally and rear their offspring to weaning Standardization of litter sizes is optional.

(B) If standardization is performed, the following procedure should be used On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four males and four females per litter or five males and five females per litter Selective elimination of pups, i e based upon body weight, is not appropriate Whenever the number of male or female pups prevents having four (or five) of each sex per litter, partial adjustment (for example, five

males and three females, or four males and six females) is acceptable Adjustments are not appropriate for litters of eight pups or less

(4) **Observation of animals**—(i) **Parental.** (A) Throughout the test period, each animal should be observed at least once daily, considering the peak period of anticipated effects after dosing Mortality, moribundity, pertinent behavioral changes, signs of difficult or prolonged parturition, and all signs of overt toxicity should be recorded at this cageside examination In addition, thorough physical examinations should be conducted weekly on each animal

(B) Parental animals (P and F1) should be weighed on the first day of dosing and weekly thereafter Parental females (P and F1) should be weighed at a minimum on approximately gestation days 0, 7, 14, and 21, and during lactation on the same days as the weighing of litters

(C) During the premating and gestation periods, food consumption should be measured weekly at a minimum Water consumption should be measured weekly at a minimum if the test substance is administered in the water

(D) Estrous cycle length and pattern should be evaluated by vaginal smears for all P and F1 females during a minimum of 3 weeks prior to mating and throughout cohabitation, care should be taken to prevent the induction of pseudopregnancy

(E) For all P and F1 males at termination, sperm from one testis and one epididymis should be collected for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, sperm from the cauda epididymis (or vas deferens) should be collected for evaluation of sperm motility and sperm morphology

(*1*) The total number of homogenization-resistant testicular sperm and cauda epididymal sperm should be enumerated (see paragraphs (g)(3) and (g)(13) of this guideline) Cauda sperm reserves can be derived from the concentration and volume of sperm in the suspension used to complete the qualitative evaluations, and the number of sperm recovered by subsequent mincing and/or homogenizing of the remaining cauda tissue Enumeration in only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated

(*2*) An evaluation of epididymal (or vas deferens) sperm motility should be performed Sperm should be recovered while minimizing damage (refer to paragraph (g)(13) of this guideline), and the percentage of progressively motile sperm should be determined either subjectively or objectively For objective evaluations, an acceptable counting chamber of sufficient depth can be used to effectively combine the assessment of motility with sperm count and sperm morphology When computer-assisted

motion analysis is performed (refer to paragraph (g)(13) of this guideline), the derivation of progressive motility relies on user-defined thresholds for average path velocity and straightness or linear index If samples are videotaped, or images otherwise recorded, at the time of necropsy, subsequent analysis of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed, in that case, the lower dose groups should also be evaluated In the absence of a video or digital image, all samples in all treatment groups should be analyzed at necropsy

(*3*) A morphological evaluation of an epididymal (or vas deferens) sperm sample should be performed Sperm (at least 200 per sample) should be examined as fixed, wet preparations (refer to paragraphs (g)(7) and (g)(13) of this guideline) and classified as either normal (both head and midpiece/tail appear normal) or abnormal Examples of morphologic sperm abnormalities would include fusion, isolated heads, and misshapen heads and/or tails Evaluation of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed, in that case, the lower dose groups should also be evaluated

(ii) **Offspring.** (A) Each litter should be examined as soon as possible after delivery (lactation day 0) to establish the number and sex of pups, stillbirths, live births, and the presence of gross anomalies Pups found dead on day 0 should be examined for possible defects and cause of death

(B) Live pups should be counted, sexed, and weighed individually at birth, or soon thereafter, at least on days 4, 7, 14, and 21 of lactation, at the time of vaginal patency or balanopreputial separation, and at termination

(C) The age of vaginal opening and preputial separation should be determined for F1 weanlings selected for mating If there is a treatment-related effect in F1 sex ratio or sexual maturation, anogenital distance should be measured on day 0 for all F2 pups

(5) **Termination schedule.** (i) All P and F1 adult males and females should be terminated when they are no longer needed for assessment of reproductive effects

(ii) F1 offspring not selected for mating and all F2 offspring should be terminated at comparable ages after weaning

(6) **Gross necropsy.** (i) At the time of termination or death during the study, all parental animals (P and F1) and when litter size permits at least three pups per sex per litter from the unselected F1 weanlings and the F2 weanlings should be examined macroscopically for any structural abnormalities or pathological changes Special attention should be paid to the organs of the reproductive system

(ii) Dead pups or pups that are terminated in a moribund condition should be examined for possible defects and/or cause of death

(iii) At the time of necropsy, a vaginal smear should be examined to determine the stage of the estrous cycle The uteri of all cohabited females should be examined, in a manner which does not compromise histopathological evaluation, for the presence and number of implantation sites

(7) **Organ weights.** (i) At the time of termination, the following organs of all P and F1 parental animals should be weighed·

(A) Uterus (with oviducts and cervix), ovaries

(B) Testes, epididymides (total weights for both and cauda weight for either one or both), seminal vesicles (with coagulating glands and their fluids), and prostate

(C) Brain, pituitary, liver, kidneys, adrenal glands, spleen, and known target organs

(ii) For F1 and F2 weanlings that are examined macroscopically, the following organs should be weighed for one randomly selected pup per sex per litter

(A) Brain

(B) Spleen and thymus

(8) **Tissue preservation.** The following organs and tissues, or representative samples thereof, should be fixed and stored in a suitable medium for histopathological examination

(i) For the parental (P and F1) animals

(A) Vagina, uterus with oviducts, cervix, and ovaries

(B) One testis (preserved in Bouins fixative or comparable preservative), one epididymis, seminal vesicles, prostate, and coagulating gland

(C) Pituitary and adrenal glands

(D) Target organs, when previously identified, from all P and F1 animals selected for mating

(E) Grossly abnormal tissue

(ii) For F1 and F2 weanlings selected for macroscopic examination. Grossly abnormal tissue and target organs, when known

(9) **Histopathology—(i) Parental animals.** Full histopathology of the organs listed in paragraph (e)(8)(i) of this guideline should be performed

for ten randomly chosen high dose and control P and F1 animals per sex, for those animals that were selected for mating Organs demonstrating treatment-related changes should also be examined for the remainder of the high-dose and control animals and for all parental animals in the low- and mid-dose groups Additionally, reproductive organs of the low- and mid-dose animals suspected of reduced fertility, e g , those that failed to mate, conceive, sire, or deliver healthy offspring, or for which estrous cyclicity or sperm number, motility, or morphology were affected, should be subjected to histopathological evaluation Besides gross lesions such as atrophy or tumors, testicular histopathological examination should be conducted in order to to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen (refer to paragraph (g)(11) of this guideline) Examination of the intact epididymis should include the caput, corpus, and cauda, which can be accomplished by evaluation of a longitudinal section, and should be conducted in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium The postlactational ovary should contain primordial and growing follicles as well as the large corpora lutea of lactation Histopathological examination should detect qualitative depletion of the primordial follicle population A quantitative evaluation of primordial follicles should be conducted for F1 females, the number of animals, ovarian section selection, and section sample size should be statistically appropriate for the evaluation procedure used

Examination should include enumeration of the number of primordial follicles, which can be combined with small growing follicles (see paragraphs (g)(1) and (g)(2) of this guideline), for comparison of treated and control ovaries

(ii) **Weanlings.** For F1 and F2 weanlings, histopathological examination of treatment-related abnormalities noted at macroscopic examination should be considered, if such evaluation were deemed appropriate and would contribute to the interpretation of the study data.

(f) **Data and reporting—**(1) **Treatment of results.** Data should be reported individually and summarized in tabular form, showing for each test group the types of change and the number of animals displaying each type of change

(2) **Evaluation of study results.** (i) An evaluation of test results, including the statistical analysis, should be provided This should include an evaluation of the relationship, or lack thereof, between the exposure of the animals to the test substance and the incidence and severity of all abnormalities

(ii) When appropriate, historical control data should be used to enhance interpretation of study results Historical data, when used, should be compiled, presented, and analyzed in an appropriate and relevant manner In order to justify its use as an analytical tool, information such as the dates of study conduct, the strain and source of the animals, and the vehicle and route of administration should be included

(iii) Statistical analysis of the study findings should include sufficient information on the method of analysis, so that an independent reviewer/ statistician can reevaluate and reconstruct the analysis

(iv) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered

(3) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J and 40 CFR part 160, subpart J, the following specific information should be reported Both individual and summary data should be presented

(i) Species and strain

(ii) Toxic response data by sex and dose, including indices of mating, fertility, gestation, birth, viability, and lactation, offspring sex ratio, precoital interval, including the number of days until mating and the number of estrous periods until mating, and duration of gestation calculated from day 0 of pregnancy The report should provide the numbers used in calculating all indices

(iii) Day (week) of death during the study or whether animals survived to termination, date (age) of litter termination

(iv) Toxic or other effects on reproduction, offspring, or postnatal growth

(v) Developmental milestone data (mean age of vaginal opening and preputial separation, and mean anogenital distance, when measured)

(vi) An analysis of P and F1 females cycle pattern and mean estrous cycle length.

(vii) Day (week) of observation of each abnormal sign and its subsequent course

(viii) Body weight and body weight change data by sex for P, F1, and F2 animals.

(ix) Food (and water, if applicable) consumption, food efficiency (body weight gain per gram of food consumed), and test material consumption for P and F1 animals, except for the period of cohabitation

(x) Total cauda epididymal sperm number, homogenization-resistant testis spermatid number, number and percent of progressively motile sperm, number and percent of morphologically normal sperm, and number and percent of sperm with each identified anomaly

(xi) Stage of the estrous cycle at the time of termination for P and F1 parental females

(xii) Necropsy findings

(xiii) Implantation data and postimplantation loss calculations for P and F1 parental females

(xiv) Absolute and adjusted organ weight data

(xv) Detailed description of all histopathological findings

(xvi) Adequate statistical treatment of results

(xvii) A copy of the study protocol and any amendments should be included

(g) **References.** The following references should be consulted for additional background information on this test guideline

(1) Bolon, B et al Differential follicle counts as a screen for chemically induced ovarian toxicity in mice results from continuous breeding bioassays *Fundamental and Applied Toxicology* 39 1-10 (1997)

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(4) Heindel, J J and R E Chapin, (eds) Part B Female Reproductive Systems, *Methods in Toxicology*, Academic, Orlando, FL (1993)

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(6) Korenbrot, C C *et al* Preputial separation as an external sign of pubertal development in the male rat *Biology of Reproduction* 17:298–303 (1977)

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(10) Pederson, T and H Peters Proposal for classification of oocytes and follicles in the mouse ovary *Journal of Reproduction and Fertility* 17 555–557 (1988)

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(12) Sadleir, R M F S Cycles and seasons, In *Reproduction in Mammals*· I Germ Cells and Fertilization, C R Auston and R V Short (eds), Cambridge, NY (1979)

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(14) Smith, B J *et al* Comparison of random and serial sections in assessment of ovarian toxicity *Reproductive Toxicology* 5 379–383 (1991)

(15) Thomas, J A Toxic responses of the reproductive system. In· *Casarett and Doull's Toxicology*, M O Amdur, J Doull, and C.D Klaassen (eds), Pergamon, NY (1991)

(16) U.S Environmental Protection Agency OPP Guideline 83–4 Reproductive and Fertility Effects Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation Human and Domestic Animals Office of Pesticides and Toxic Substances, Washington, DC, EPA–540/9–82–025 (1982)

(17) U S Environmental Protection Agency Subpart E—Specific Organ/Tissue Toxicity, 40 CFR 798 4700 Reproduction and Fertility Effects

(18) U S Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870 3250, 90-Day Dermal Toxicity, July 1998

(19) U S Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870 3465, 90-Day Inhalation Toxicity, July 1998

(20) U S Environmental Protection Agency Reproductive Toxicity Risk Assessment Guidelines **Federal Register** 61 FR 56274–56322 (1996)

(21) Working, P K and M Hurtt Computerized videomicrographic analysis of rat sperm motility *Journal of Andrology* 8 330–337 (1987)

(22) Zenick, H et al Assessment of male reproductive toxicity a risk assessment approach In *Principles and Methods of Toxicology*, A W Hayes (ed.), Raven, NY (1994)

United States
Environmental Protection
Agency

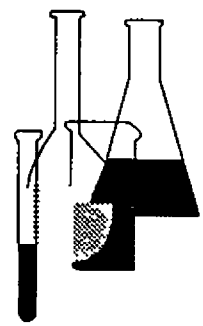
Prevention Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–210
August 1998

EPA

Health Effects Test Guidelines

OPPTS 870.4100
Chronic Toxicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in title 40, chapter I, subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq.*)

Final Guideline Release: This guideline is available from the U.S Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132 This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http //www.epa.gov/epahome/research htm) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines."

OPPTS 870.4100 Chronic toxicity.

(a) **Scope**—(1) **Applicability** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 798 3260 Chronic Toxicity, OPP 83–1 Chronic Feeding—Two Species, Rodent and Nonrodent (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 452 Chronic Toxicity studies

(b) **Purpose.** The objective of a chronic toxicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure A chronic toxicity study should generate data from which to identify the majority of chronic effects and to define long-term dose-response relationships The design and conduct of chronic toxicity tests should allow for the detection of general toxic effects, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathological) effects

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline.

Chronic toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral, dermal, or inhalation routes of exposure

Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissue

Dose in a chronic toxicity study is the amount of test substance administered daily via the oral, dermal or inhalation routes for a period of at least 12 months Dose is expressed as weight of the test substance (grams, milligrams) per unit body weight (BW) of test animal (milligram per kilogram), or as weight of the test substance in parts per million (ppm) in food or drinking water per day For inhalation exposure, dose is expressed as weight of the test substance per unit volume of air (milligrams per liter) or as parts per million per day For dermal exposure, dose is expressed as weight of the test substance (grams, milligrams) per unit body weight of the test animal (milligrams per kilogram) or as weight of the substance per unit of surface area (milligrams per square centimeter) per day

No-observed-effect-level (NOEL) is the maximum dose used in a study which produces no adverse effects The NOEL is usually expressed

in terms of the weight of a test substance given daily per unit weight of test animal (milligrams per kilogram per day)

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg BW (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, a full study using three dose levels might not be necessary

(e) **Test procedures—(1) Animal selection—(i) Species and strain** Testing should be performed with two mammalian species, one a rodent and the other a nonrodent The rat is the preferred rodent species and the dog is the preferred nonrodent species Commonly used laboratory strains should be employed If other mammalian species are used, the tester should provide justification/reasoning for their selection

(ii) **Age/weight.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization.

(B) Dosing of rodents should generally begin no later than 8 weeks of age.

(C) Dosing of dogs should begin between 4 and 6 months of age and in no case later than 9 months of age

(D) At commencement of the study, the weight variation of animals used should be within 20 percent of the mean weight for each sex.

(E) Studies using prenatal or neonatal animals may be recommended under special conditions.

(iii) **Sex.** (A) Equal numbers of animals of each sex should be used at each dose level

(B) Females should be nulliparous and nonpregnant

(iv) **Numbers.** (A) For rodents, at least 40 animals (20 males and 20 females) and for nonrodents (dogs) at least 8 animals (4 females and 4 males) should be used at each dose level and concurrent control group.

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed during the course of the study

(C) The number of animals at the termination of the study must be adequate for a meaningful and valid statistical evaluation of chronic ef-

fects The Agency must be notified if excessive early deaths or other problems are encountered that might compromise the integrity of the study

(D) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required

(E) Each animal should be assigned a unique identification number Dead animals, their preserved organs and tissues, and microscopic slides should be identified by reference to the unique numbers assigned.

(v) **Husbandry.** (A) Rodents may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal The biological properties of the test substance or toxic effects (e g , morbidity, excitability) may indicate a need for individual caging. Rodents should be housed individually in dermal studies and during exposure in inhalation studies Caging should be appropriate to the nonrodent species However, it is recommended that dogs are housed individually

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C

(C) The relative humidity of the experimental animal rooms should be 50 ± 20 percent

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark.

(E) Control and test animals should be fed from the same batch and lot. The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. Animals should be fed and watered ad libitum with food replaced at least weekly

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least 5 days is recommended

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed it should not elicit toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance It is recommended that wherever possible the use of an aqueous solution be the first choice, followed by consideration of solution in oil, and finally, solution in other vehicles

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation

of the study, there should be a characterization of the test substance, including the purity of the test compound, and, if technically feasible, the names and quantities of contaminants and impurities

(iii) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture

(3) **Control groups.** A concurrent control group is required This group should be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required

(4) **Satellite group.** A satellite group of 40 animals (20 animals per sex) for rodents and 8 animals (4 animals per sex) for nonrodents may be treated with the high-dose level for 12 months and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment of appropriate length, normally not less than 28 days. In addition, a control group of 40 animals (20 animals per sex) for rodents and 8 animals (4 animals per sex) for nonrodents should be added to the satellite study.

(5) **Dose levels and dose selections.** (i) In chronic toxicity tests, it is desirable to determine a dose-response relationship as well as a NOEL Therefore, at least three dose levels with a control group and, where appropriate, a vehicle control (corresponding to the concentration of the vehicle at the highest exposure level) should be used Dose levels should be spaced to produce a gradation of effects A rationale must be provided for the doses selected

(ii) The highest-dose level should elicit signs of toxicity without substantially altering the normal life span of the animal The highest dose should be determined based on the findings from a 90-day study to ensure that the dose used is adequate to assess the chronic toxicity of the test substance Thus, the selection of the highest dose to be tested is dependent upon changes observed in several toxicological parameters in subchronic studies The highest dose tested need not exceed 1,000 mg/kg/day If dermal application of the test substance produces severe skin irritation, then it may be necessary either to terminate the study and choose a lower high-dose level or to reduce the dose level Gross criteria for defining severe irritation would include ulcers, fissures, exudate/crust(eschar), dead tissue,

or anything leading to destruction of the functional integrity of the epidermis (e g caking, open sores, fissuring, eschar) Histological criteria for defining severe irritation would include follicular and interfollicular crust microulcer, mild/moderate degeneration/necrosis, moderate/marked epidermal edema, marked dermal edema, and marked inflammation

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects

(iv) The lowest-dose level should produce no evidence of toxicity

(6) **Administration of the test substance.** The three main routes of administration are oral, dermal, and inhalation The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans

(i) **Oral studies.** Ideally, the animals should be dosed by gavage or with capsules on a 7-day per week basis for a period of at least 12 months. However, based primarily on practical considerations, dosing by gavage or capsules on a 5-day per week schedule is acceptable If the test substance is administered via in the drinking water or mixed in the diet, exposure should be on a 7-day per week basis

(ii) **Dermal studies.** (A) Preparation of animal skin Shortly before testing, fur should be clipped from not less than 10 percent of the body surface area for application of the test substance In order to dose approximately 10 percent of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half way down the flank on each side of the animal should be shaved Shaving should be carried out approximately 24 hours before dosing Repeated clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care should be taken to avoid abrading the skin which could alter its permeability

(B) Preparation of test substance Liquid test substances are generally used undiluted, except as indicated in paragraph (e)(5)(ii) of this guideline. Solids should be pulverized when possible The substance should be moistened sufficiently with water or, when necessary, with a suitable vehicle to ensure good contact with the skin When a vehicle is used, the influence of the vehicle on toxicity of, and penetration of the skin by, the test substance should be taken into account The volume of application should be kept constant, e g less than 100 µL for the mouse and less than 300 µL for the rat Different concentrations of test solution should be prepared for different dose levels

(C) Administration of test substance The duration of exposure should be at least for 12 months Ideally the animals should be treated with test substance for at least 6 h/day on a 7-day per week basis However, based on practical considerations, application on a 5-day per week basis is ac-

ceptable Dosing should be conducted at approximately the same time each day The test substance should be applied uniformly over the treatment site The surface area covered may be less for highly toxic substances As much of the area should be covered with as thin and uniform a film as possible For rats, the test substance may be held in contact with the skin with a porous gauze dressing and nonirritating tape if necessary The test site should be further covered in a suitable manner to retain the gauze dressing plus test substance and to ensure that the animals cannot ingest the test substance. The application site should not be covered when the mouse is the species of choice The test substance may be wiped from the skin after the six-hour exposure period to prevent ingestion

(iii) *Inhalation studies* (A) The animals should be exposed to the test substance for 6 h/day on a 7-day per week basis, for a period of at least 12 months However, based primarily on practical considerations, exposure for 6 hours per day on a 5-day per week basis is acceptable.

(B) The animals should be tested in dynamic inhalation equipment designed to sustain a minimum air flow of 10 air changes per hour, an adequate oxygen content of at least 19 percent, and uniform conditions throughout the exposure chamber Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding areas It is not normally necessary to measure chamber oxygen concentration if airflow is adequate

(C) The selection of a dynamic inhalation chamber should be appropriate for the test substance and test system When a whole body chamber is used, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance To ensure stability of a chamber atmosphere, the total volume occupied by the test animals should not exceed 5 percent of the volume of the test chamber It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order to minimize oral exposures due to animals licking compound off their fur The animals should be acclimated and heat stress minimized

(D) The temperature at which the test is performed should be maintained at 22 ± 2 °C The relative humidity should be maintained between 40–60 percent, but in certain instances (e g , use of water vehicle) this may not be practicable

(E) The rate of air flow should be monitored continuously but recorded at least three times during the exposure

(F) Temperature and humidity should be monitored continuously but should be recorded at least every 30 min

(G) The actual concentrations of the test substance should be measured in the breathing zone During the exposure period, the actual con-

centrations of the test substance should be held as constant as practicable, monitored continuously or intermittently depending on the method of analysis Chamber concentration may be measured using gravimetric or analytical methods, as appropriate If trial run measurements are reasonably consistent (± 10 percent for liquid aerosol, gas, or vapor, ± 20 percent for dry aerosol), then two measurements should be sufficient If measurements are not consistent, three to four measurements should be taken Whenever the test substance is a formulation, or it is necessary to formulate the test substance with a vehicle for aerosol generation, the analytical concentration must be reported for the total formulation, and not just for the active ingredient (AI). If, for example, a formulation contains 10 percent AI and 90 percent inerts, a chamber of analytical limit concentration of 2 mg/L would consist of 0 2 mg/L of the AI It is not necessary to analyze inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation, the grounds for this conclusion must be provided in the study report If there is some difficulty measuring chamber analytical concentration due to precipitation, nonhomogeneous mixtures, volatile components, or other factors, additional analysis of inert components may be necessary

(H) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations with respect to particle size. The mass median aerodynamic diameter (MMAD) particle size range should be between 1–3 μm. The particle size of hygroscopic materials should be small enough when dry to assure that the size of the swollen particle will still be within the 1–3 μm range Measurements of aerodynamic particle size in the animal's breathing zone should be measured during a trial run. If MMAD values for each exposure level are within 10 percent of each other, then two measurements during the exposures should be sufficient If pretest measurements are not within 10 percent of each other, three to four measurements should be taken

(I) Feed should be withheld during exposure Water may also be withheld during exposure

(7) **Observation period.** (i) Animals should be observed for a period of at least 12 months

(ii) Animals in a satellite group (if used) scheduled for follow-up observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects

(8) **Observation of animals.** (i) Observations should be made at least twice each day for morbidity and mortality Appropriate actions should be taken to minimize loss of animals to the study (e g , necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals) General clinical observations should be made at least once a day, preferably at the same time each day, taking into consid-

eration the peak period of anticipated effects after dosing The clinical condition of the animal should be recorded

(ii) A careful clinical examination should be made at least once prior to the initiation of treatment (to allow for within subject comparisons) and once weekly during treatment in all animals These observations should be made outside the home cage, preferably in a standard arena, and at similar times on each occasion Effort should be made to ensure that variations in the observation conditions are minimal. Observations should be detailed and carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e g , lacrimation, piloerection, pupil size, unusual respiratory pattern) Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e g , excessive grooming, repetitive circling) or bizarre behavior (e g , self-mutilation, walking backwards) should be recorded.

(iii) Once, near the end of the first year of the exposure period and in any case not earlier than in month 11, assessment of motor activity, grip strength, and sensory reactivity to stimuli of different types (e g , visual, auditory, and proprioceptive stimuli) should be conducted in rodents Further details of the procedures that could be followed are described in the references listed under paragraphs (h)(2), (h)(6), (h)(8), (h)(9), (h)(10), and (h)(17) of this guideline

(iv) Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits.

(v) Exceptionally, functional observations may be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with functional test performance

(vi) Body weights should be recorded individually for all animals once prior to the administration of the test substance, once a week during the first 13 weeks of study and at least once every 4 weeks thereafter, unless signs of clinical toxicity suggest more frequent weighing to facilitate monitoring of health status

(vii) Measurements of feed consumption should be determined weekly during the first 13 weeks of the study and at approximately monthly intervals thereafter unless health status or body weight changes dictate otherwise. Measurements of water consumption should be determined at the same intervals if the test substance is administered in the drinking water

(viii) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible All survivors should be sacrificed at the end of the study period

(9) **Clinical pathology.** Hematology, clinical chemistry, and urinalysis should be performed on 10 rats per sex per group, and on all nonrodents In rodents, the parameters should be examined at approximately 6 month intervals during the conduct of the study and at termination If possible, these collections should be from the same animals at each interval In nonrodents, the parameters should be examined once or twice prior to initiation of treatment, at 6-month intervals during the conduct of the study, and at termination If hematological and biochemical effects were seen in the subchronic study, testing should also be performed at 3 months Overnight fasting of animals prior to blood sampling is recommended

(i) **Hematology.** The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time.

(ii) **Clinical chemistry.** (A) Parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity

(B) The recommended clinical chemistry determinations are potassium, sodium, calcium (nonrodent), phosphorus (nonrodent), chloride (nonrodent), glucose, total cholesterol, urea nitrogen, creatinine, total protein, total bilirubin (nonrodent), and albumin More than two hepatic enzymes, (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, or gamma glutamyl transpeptidase) should also be measured Measurements of addtional enzymes (of hepatic or other origin) and bile acids, may also be useful

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated.

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include calcium, phosphorus, fasting triglycerides, hormones, methemoglobin, and cholinesterases

(iii) **Urinalysis.** Urinalysis for rodents should be performed at the end of the study using timed urine collection Urinalysis for nonrodents should be performed prior to treatment, midway through treatment and at the end of the study using timed urine collection Urinalysis determina-

tions include appearance, volume, osmolality or specific gravity, pH, protein, glucose, and blood/blood cells

(10) **Ophthalmological examination.** Examinations should be made of all animals using an ophthalmoscope or equivalent device prior to the administration of the test substance and at termination of the study on 10 rats of each sex in the high-dose and control groups and preferably in all nonrodents, but at least the control and high-dose groups should be examined If changes in eyes are detected, all animals should be examined

(11) **Gross necropsy.** (i) All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents

(ii) At least the liver, kidneys, adrenals, testes, epididymides, ovaries, uterus, nonrodent thyroid (with parathyroid), spleen, brain, and heart should be weighed wet as soon as possible after dissection to avoid drying The lungs should be weighed if the test substance is administered by the inhalation route

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder (when present) .

(B) Nervous system—brain (multiple sections, including cerebrum, cerebellum and medulla/pons), pituitary, peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle), spinal cord (three levels, cervical, mid-thoracic and lumbar), eyes (retina, optic nerve)

(C) Glandular system—adrenals, parathyroid, thyroid.

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose

(E) Cardiovascular/hematopoietic system—aorta, heart, bone marrow (and/or fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland

(G) Other—all gross lesions and masses, skin

(iv) In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved

In dermal studies, skin from treated and adjacent control skin sites should be examined and preserved

(v) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues The proper inflation and fixation of the lungs in inhalation studies is considered essential for appropriate and valid histopathological examination

(vi) Information from clinical pathology and other in-life data should be considered before microscopic examination, since they may provide significant guidance to the pathologist

(12) **Histopathology.** (i) The following histopathology should be performed

(A) Full histopathology on the organs and tissues (listed under paragraph (e)(11)(iii) of this guideline) of all rodents and nonrodents in the control and high-dose groups, and all rodents and nonrodents that died or were killed during the study The examination should be extended to all animals in all dosage groups if treatment-related changes are observed in the high-dose group

(B) All gross lesions in all animals

(C) Target tissues in all animals

(ii) If the results show substantial alteration of the animal's normal life span, or other effects that might compromise the significance of the data, the next lower levels should be examined fully as described in paragraph (e)(12)(i) of this guideline

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming.

(f) **Data and reporting—**(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) When applicable, all observed results (quantitative and qualitative) should be evaluated by an appropriate statistical method Any generally accepted statistical methods may be used, the statistical methods including significance criteria should be selected during the design of the study

(2) **Evaluation of study results.** The findings of a chronic toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects as well as the necropsy and histopathological findings The evaluation will include the relationship between the dose of the test substance and the presence, incidence, and severity of abnormalities (including behavioral and clinical abnormalities) gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects

(3) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, 40 CFR part 160, and the OECD Principles of GLP (ISBN 92–64–12367–9), the following specific information should be reported·

(i) Test substance characterization should include

(A) Chemical identification

(B) Lot or batch number

(C) Physical properties

(D) Purity/impurities

(ii) Identification and composition of any vehicle used

(iii) Test system should contain data on

(A) Species and strain of animals used and rationale for selection if other than that recommended

(B) Age including body weight data and sex

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(D) Identification of animal diet

(E) Acclimation period

(iv) Test procedure should include the following data·

(A) Method of randomization used

(B) Full description of experimental design and procedure

(C) Dose regimen including levels, methods, and volume

(v) Test results

(A) Group animal data Tabulation of toxic response data by species, strain, sex and exposure level for

(*1*) Number of animals exposed

(*2*) Number of animals showing signs of toxicity

(*3*) Number of animals dying

(B) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(*1*) Time of death during the study or whether animals survived to termination.

(*2*) Time of observation of each abnormal sign and its subsequent course

(*3*) Body weight data

(*4*) Feed and water (if collected) consumption data

(*5*) Achieved dose (mg/kg/day) as a time-weighted average if the test substance is administered in the diet or drinking water

(*6*) Results of ophthalmological examinations

(*7*) Results of hematological tests performed

(*8*) Results of clinical chemistry tests performed

(*9*) Urinalysis tests performed and results

(*10*) Results of observations made

(*11*) Necropsy findings, including absolute and relative (to body weight) organ weight data

(*12*) Detailed description of all histopathological findings

(*13*) Statistical treatment of results, where appropriate

(iv) In addition, for inhalation studies the following should be reported

(A) Test conditions The following exposure conditions must be reported·

(*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulate and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described

(B) Exposure data These data should be tabulated and presented with mean values and a measure of variability (e g , standard deviation) and should include

(*1*) Airflow rates through the inhalation equipment

(*2*) Temperature and humidity of air

(*3*) Actual (analytical or gravimetric) concentration in the breathing zone

(*4*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air)

(*5*) Particle size distribution, calculated MMAD, and geometric standard deviation (GSD)

(*6*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable) and the efforts taken to comply with this aspect of the guidelines

(g) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment. The study must be conducted in compliance with GLP regulations as described by the Agency (40 CFR parts 160 and 792), and the OECD principles of GLP (ISBN 92–64–12367–9)

(h) **References.** The following references should be consulted for additional background information on this test guideline

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(2) Crofton K.M , Howard J L , Moser V C , Gill M.W , Leiter L W , Tilson H A., MacPhail, R C Interlaboratory Comparison of Motor Activity Experiments· Implication for Neurotoxicological Assesments *Neurotoxicol Teratol* 13, 599–609 (1991)

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(9) Meyer O A , Tilson H A , Byrd W C , Riley M T A Method for the Routine Assessment of Fore- and Hind-Limb Grip Strength of Rats and Mice. *Neurobehav Toxicol* 1, 233–236 (1979)

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(16) Toxicity and Clinical Trial Subcommittee, Committee on Safety of Medicine (November 1977)

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(18) United States Pharmaceutical Manufacturers Association Guidelines for the Assessment of Drug and Medical Device Safety in Animals (1977)

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United States
Environmental Protection
Agency

Prevention Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–211
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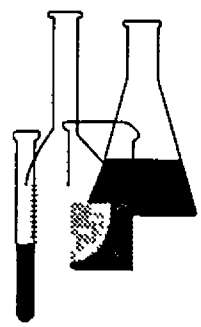
Health Effects Test Guidelines OPPTS 870.4200 Carcinogenicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

Final Guideline Release: This guideline is available from the U S Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132 This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http //www epa gov/epahome/research htm) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines "

OPPTS 870.4200 Carcinogenicity.

(a) **Scope**—(1) **Applicability** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 798 3300 Oncogenicity, OPP 83–2 Carcinogenicity—Two Species, Rat and Mouse Preferred (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982, and OECD 451 Carcinogenicity Studies

(b) **Purpose.** The objective of a long-term carcinogenicity study is to observe test animals for a major portion of their life span for development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this guideline The following definitions also apply to this guideline

Carcinogenicity is the development of neoplastic lesions as a result of the repeated daily exposure of experimental animals to a chemical by the oral, dermal, or inhalation routes of exposure

Cumulative toxicity is the adverse effects of repeated dose occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissues.

Dose in a carcinogenicity study is the amount of test substance administered via the oral, dermal or inhalation routes for a period of up to 24 months Dose is expressed as weight of the test substance (grams, milligrams) per unit body weight of test animal (milligram per kilogram), or as weight of the test substance in parts per million (ppm) in food or drinking water When exposed via inhalation, dose is expressed as weight of the test substance per unit volume of air (milligrams per liter) or as parts per million

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance

(d) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** Testing should be performed on two mammalian species Rats and mice are the species of choice because of their relatively short life spans, limited cost of maintenance, widespread use in pharmacological and toxicological studies, susceptibility to tumor induction, and the availability of inbred or sufficiently characterized strains Commonly used laboratory strains

should be used If other mammalian species are used, the tester should provide justification/reasoning for their selection

(ii) **Age/weight.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization.

(B) Dosing should generally begin no later than 8 weeks of age

(C) At commencement of the study, the weight variation of animals used should be within 20 percent of the mean weight for each sex

(D) Studies using prenatal or neonatal animals may be recommended under special conditions

(iii) **Sex.** (A) Equal numbers of animals of each sex should be used at each dose level

(B) Females should be nulliparous and nonpregnant

(iv) **Numbers.** (A) At least 100 rodents (50 males and 50 females) should be used at each dose level and concurrent control group.

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed during the course of the study.

(C) For a meaningful and valid statistical evaluation of long term exposure and for a valid interpretation of negative results, the number of animals in any group should not fall below 50 percent at 15 months in mice and 18 months in rats Survival in any group should not fall below 25 percent at 18 months in mice and 24 months in rats

(D) The use of adequate randomization procedures for the proper allocation of animals to test and control groups is required to avoid bias

(E) Each animal should be assigned a unique identification number. Dead animals, their preserved organs and tissues, and microscopic slides should be identified by reference to the unique numbers assigned.

(v) **Husbandry.** (A) Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal The biological properties of the test substance or toxic effects (e.g , morbidity, excitability) may indicate a need for individual caging Animals should be housed individually in dermal studies and during exposure in inhalation studies

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C

(C) The relative humidity of the experimental animal rooms should be 50 ± 20 percent.

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark

(E) Control and test animals should be fed from the same batch and lot The feed should be analyzed to assure uniform distribution and adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test Animals should be fed and watered ad libitum with food replaced at least weekly

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least five days is recommended

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, it should not elicit toxic effects itself It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of solution in oil, and finally solution in other vehicles

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound, and, if possible, the name and quantities of contaminants and impurities

(iii) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(3) **Control groups.** A concurrent control group (50 males and 50 females) is required. This group should be untreated or if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known, both untreated and vehicle control groups are required

(4) **Dose levels and dose selection.** (i) For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group Dose levels should be spaced to produce a gradation of effects. A rationale for the doses selected must be provided

(ii) The highest-dose level should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors The highest dose should be determined based on the findings from a 90–day study to ensure that the dose used is adequate to asses the carcinogenic potential of the test substance Thus, the selection of the highest dose to be tested is dependent upon changes observed in several toxicological parameters in subchronic studies The highest dose tested need not exceed 1,000 mg/kg/day

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects

(iv) The lowest-dose level should produce no evidence of toxicity

(v) For skin carcinogenicity studies, when toxicity to the skin is a determining factor, the highest dose selected should not destroy the functional integrity of the skin, the intermediate doses should be a minimally irritating dose, and the low dose should be the highest nonirritating dose

(vi) The criteria for selecting the dose levels for skin carcinogenicity studies, based on gross and histopathologic dermal lesions, are as follows

(A) Gross criteria for reaching the high dose

(*1*) Erythema (moderate)

(*2*) Scaling.

(*3*) Edema (mild)

(*4*) Alopecia.

(*5*) Thickening

(B) Histologic criteria for reaching the high-dose·

(*1*) Epidermal hyperplasia

(*2*) Epidermal hyperkeratosis

(*3*) Epidermal parakeratosis

(*4*) Adnexal atrophy/hyperplasia

(*5*) Fibrosis.

(*6*) Spongiosis (minimal-mild)

(*7*) Epidermal edema (minimal-mild)

(*8*) Dermal edema (minimal-moderate)

(*9*) Inflammation (moderate)

(C) Gross criteria for exceeding the high-dose

(*1*) Ulcers, fissures

(*2*) Exudate/crust (eschar)

(*3*) nonviable (dead) tissues

(*4*) Anything leading to destruction of the functional integrity of the epidermis (e g , caking, fissuring, open sores, eschar)

(D) Histologic criteria for exceeding the high-dose

(*1*) Crust (interfollicular and follicular)

(*2*) Microulcer

(*3*) Degeneration/necrosis (mild to moderate)

(*4*) Epidermal edema (moderate to marked)

(*5*) Dermal edema (marked)

(*6*) Inflammation (marked)

(5) **Administration of the test substance.** The three main routes of administration are oral, dermal, and inhalation The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

(i) **Oral studies.** If the test substance is administered by gavage, the animals are dosed with the test substance on a 7–day per week basis for a period of at least 18 months for mice and hamsters and 24 months for rats However, based primarily on practical considerations, dosing by gavage on a 5–day per week basis is acceptable If the test substance is administered in the drinking water or mixed in the diet, then exposure should be on a 7–day per week basis

(ii) **Dermal studies.** (A) The animals should be treated with the test substance for at least 6 h/day on a 7-day per week basis for a period of at least 18 months for mice and hamsters and 24 months for rats However, based primarily on practical considerations, application on a 5-day per week basis is acceptable Dosing should be conducted at approximately the same time each day

(B) Fur should be clipped weekly from the dorsal area of the trunk of the test animals Care should be taken to avoid abrading the skin which could alter its permeability A minimum of 24 hours should be allowed for the skin to recover before the next dosing of the animal

(C) Preparation of test substance Liquid test substances are generally used undiluted, except as indicated in paragraph (e)(4)(vi) of this guideline

Solids should be pulverized when possible The substance should be moistened sufficiently with water or, when necessary, with a suitable vehicle to ensure good contact with the skin When a vehicle is used, the influence of the vehicle on toxicity of, and penetration of the skin by, the test substance should be taken into account The volume of application should be kept constant, e g less than 100 µL for the mouse and less than 300 µL for the rat. Different concentrations of test solution should be prepared for different dose levels

(D) The test substance should be applied uniformly over a shaved area which is approximately 10 percent of the total body surface area In order to dose approximately 10 percent of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half way down the flank on each side of the animal should be shaved. With highly toxic substances, the surface area covered may be less, but as much of the area as possible should be covered with as thin and uniform a film as practical

(E) During the exposure period, the application site should not be covered when mice or hamsters are the species of choice. For rats, the test substance may be held in contact with the skin with a porous gauze dressing and nonirritating tape if necessary The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance The test substance may be wiped from the skin after the 6-hour exposure to prevent ingestion

(iii) **Inhalation studies.** (A) The animals should be exposed to the test substance for 6 h/day on a 7-day per week basis, for a period of at least 18 months in mice and 24 months in rats However, based primarily on practical considerations, exposure for 6 h/day on a 5–day per week basis is acceptable

(B) The animals should be tested in dynamic inhalation equipment designed to sustain a minimum air flow of 10 air changes per hour, an adequate oxygen content of at least 19 percent, and uniform conditions throughout the exposure chamber Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding areas.

(C) The selection of a dynamic inhalation chamber should be appropriate for the test substance and test system Where a whole body chamber is used to expose animals to an aerosol, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance To ensure stability of a chamber atmosphere, the total volume occupied by the test animals should not exceed 5 percent of the volume of the test chamber It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order

to minimize oral exposures due to animals licking compound off their fur Heat stress should be minimized

(D) The temperature at which the test is performed should be maintained at 22 ± 20 °C The relative humidity should be maintained between 40 to 60 percent, but in certain instances (e g , tests of aerosols, use of water vehicle) this may not be practicable

(E) The rate of air flow should be monitored continuously but recorded at least three times during exposure

(F) Temperature and humidity should be monitored continuously but should be recorded at least every 30 minutes

(G) The actual concentrations of the test substance should be measured in the breathing zone During the exposure period, the actual concentrations of the test substance should be held as constant as practicable, monitored continuously or intermittently depending on the method of analysis. Chamber concentrations may be measured using gravimetric or analytical methods as appropriate If trial run measurements are reasonably consistent (± 10 percent for liquid aerosol, gas, or vapor, ± 20 percent for dry aerosol), the two measurements should be sufficient If measurements are not consistent, then three to four measurements should be taken Whenever the test substance is a formulation, or it is necessary to formulate the test substance with a vehicle for aerosol generation, the analytical concentration must be reported for the total formulation, and not just for the active ingredient (AI) If, for example, a formulation contains 10 percent AI and 90 percent inerts, a chamber of analytical limit concentration of 2 mg/L would consist of 0 2 mg/L of the AI It is not necessary to analyze inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation, the grounds for this conclusion must be provided in the study report If there is some difficulty measuring chamber analytical concentration due to precipitation, nonhomogeneous mixtures, volatile components, or other factors, additional analysis of inert components may be necessary

(H) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations with respect to particle size Measurement of aerodynamic particle size in the animals's breathing zone should be measured during a trial run. If MMAD values for each exposure level are within 10 percent of each other, then two measurements during the exposures should be sufficient If pretest measurements are not within 10 percent of each other, three to four measurements should be taken The mass median aerodynamic diameter (MMAD) particle size range should be between 1–3 μm The particle size of hygroscopic materials should be small enough when dry to assure that the size of the swollen particle will still be within the 1–3 μm range

(I) Feed should be withheld during exposure Water may also be withheld during exposure

(6) **Observation period.** It is necessary that the duration of the carcinogenicity study comprise the majority of the normal life span of the strain of animals used This time period should not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice For longer time periods, and where any other species are used, consultation with the Agency in regard to the duration of the study is advised

(7) **Observation of animals.** (i) Observations should be made at least twice each day for morbidity and mortality Appropriate actions should be taken to minimize loss of animals from the study (e g , necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals)

(ii) A careful clinical examination should be made at least once weekly Observations should be detailed and carefully recorded, preferably using explicitly defined scales Observations should include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength and stereotypies or bizarre behavior (e g., self-mutilation, walking backwards)

(iii) Body weights should be recorded individually for all animals once pretreatment, once a week during the first 13 weeks of the study and at least once every 4 weeks, thereafter, unless signs of clinical toxicity suggest more frequent weighing to facilitate monitoring of health status

(iv) Measurements of feed consumption should be determined weekly during the first 13 weeks of the study and at approximately monthly intervals thereafter unless health status or body weight changes dictate otherwise Measurements of water consumption should be determined at the same intervals if the test substance is administered in the drinking water.

(v) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible. At the end of the study period, all survivors should be sacrificed

(8) **Clinical pathology.** At 12 months, 18 months, and at terminal sacrifice, a blood smear should be obtained from all animals A differential blood count should be performed on blood smears from those animals in the highest dosage group and the controls from the terminal sacrifice If these data, or data from the pathological examination indicate a need, then the 12- and 18-month blood smears should also be examined Differential blood counts should be performed for the next lower groups if there is

a major discrepancy between the highest group and the controls If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed

(9) **Gross necropsy.** (i) A complete gross examination should be performed on all animals, including those that died during the experiment or were killed in a moribund condition

(ii) At least the liver, kidneys, adrenals, testes, epididymides, ovaries, uterus, spleen, brain and heart should be weighed wet as soon as possible after dissection to avoid drying The lungs should be weighed if the test substance is administered by the inhalation route The organs should be weighed from interim sacrifice animals as well as from at least 10 animals per sex per group at terminal sacrifice

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder (when present)

(B) Nervous system—brain (multiple sections, including cerebrum, cerebellum and medulla/pons), pituitary, peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle), spinal cord (three levels, cervical, mid-thoracic and lumbar), eyes (retina, optic nerve)

(C) Glandular system—adrenals, parathyroid, thyroid

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose.

(E) Cardiovascular/hematopoietic system—aorta, heart, bone marrow (and/or fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland

(G) Other—all gross lesions and masses, skin

(iv) In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved In dermal studies, skin from treated and adjacent control skin sites should be examined and preserved

(v) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues The proper inflation and fixation

of the lungs in inhalation studies is essential for appropriate and valid histopathological examination

(vi) Information from clinical pathology, and other in-life data should be considered before microscopic examination, since they may provide significant guidance to the pathologist

(10) **Histopathology.** (i) The following histopathology should be performed·

(A) Full histopathology on the organs and tissues listed under paragraph (d)(9)(iii) of this guideline of all animals in the control and high-dose groups and all animals that died or were killed during the study

(B) All gross lesions in all animals

(C) Target organs in all animals

(ii) If the results show substantial alteration of the animal's normal life span, the induction of effects that might affect a neoplastic response, or other effects that might compromise the significance of the data, the next lower dose levels should be examined as described under paragraph (d)(10)(i) of this guideline

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming

(e) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

(ii) When applicable, all observed results (quantitative and qualitative) should be evaluated by an appropriate statistical method Any generally accepted statistical methods may be used, the statistical methods including significance criteria should be selected during the design of the study

(2) **Evaluation of study results.** (i) The findings of a carcinogenicity study should be evaluated in conjunction with the findings of previous studies and considered in terms of the toxic effects, the necropsy and histopathological findings The evaluation should include the relationship between the dose of the test substance and the presence, incidence, and severity of abnormalities (including behavioral and clinical abnormalities),

gross lesions, identified target organs, body weight changes, effects on mortality, and any other general or specific toxic effects

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailablity of the test substance should be considered

(iii) In order for a negative test to be acceptable, it should meet the following criteria No more than 10 percent of any group is lost due to autolysis, cannibalism, or management problems, and survival in each group is no less than 50 percent at 15 months for mice and 18 months for rats Survival should not fall below 25 percent at 18 months for mice and 24 months for rats

(iv) The use of historical control data from an appropriate time period from the same testing laboratory (i e , the incidence of tumors and other suspect lesions normally occurring under the same laboratory conditions and in the same strain of animals employed in the test) is helpful for assessing the significance of changes observed in the current study.

(3) **Test report.** (i) In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, 40 CFR part 160, and the OECD Principles of GLP (ISBN 92–64–12367–9), the following specific information should be reported

(A) Test substance characterization should include

(*1*) Chemical identification

(*2*) Lot or batch number

(*3*) Physical properties

(*4*) Purity/impurities

(*5*) Identification and composition of any vehicle used

(B) Test system should contain data on

(*1*) Species and strain of animals used and rationale for selection if other than that recommended

(*2*) Age including body weight data and sex.

(*3*) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(*4*) Identification of animal diet

(*5*) Acclimation period

(C) Test procedure should include the following data

(*1*) Method of randomization used

(*2*) Full description of experimental design and procedure

(*3*) Dose regimen including levels, methods, and volume

(4) **Test results.** (i) Group animal data Tabulation of toxic response data by species, strain, sex and exposure level for

(A) Number of animals exposed

(B) Number of animals showing signs of toxicity

(C) Number of animals dying

(ii) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(A) Time of death during the study or whether animals survived to termination.

(B) Time of observation of each abnormal sign and its subsequent course

(C) Body weight data.

(D) Feed and water consumption data, when collected

(E) Achieved dose (mg/kg/day) as a time-weighted average if the test substance is administered in the diet or drinking water

(F) Results of clinical pathology when performed

(G) Necropsy findings including absolute/relative organ weight data.

(H) Detailed description of all histopathological findings

(I) Statistical treatment of results where appropriate

(J) Historical control data.

(iii) In addition, for inhalation studies the following should be reported:

(A) Test conditions The following exposure conditions must be reported

(*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulate and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described

(B) Exposure data These should be tabulated and presented with mean values and a measure of variability (e g standard deviation) and should include

(*1*) Airflow rates through the inhalation equipment

(*2*) Temperature and humidity of air

(*3*) Actual (analytical or gravimetric) concentration in the breathing zone

(*4*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air)

(*5*) Particle size distribution, calculated MMAD and geometric standard deviation (GSD)

(*6*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable) and the efforts taken to comply with this aspect of the guidelines

(f) **Quality assurance.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment The study must be conducted in compliance with the GLP regulations as described by the Agency (40 CFR parts 160 and 792) and the OECD Principles of GLP (ISBN 92–64–12367–9)

(g) **References.** The following references should be consulted for additional background information on this guideline:

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(3) Department of Health and Welfare The Testing of Chemicals for Carcinogenicity, Mutagenicity, Teratogenicity Minister of Health and Welfare Department of Health and Welfare, Canada (1975)

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(5) Food Safety Council Proposed System for Food Safety Assessment Prepared by the Scientific Committee, Food Safety Council *Food and Cosmetic Toxicology* , Vol 16, Supplement 2 (December 1978).

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(10) National Cancer Institute Report of the Subtask Group on Carcinogen Testing to the Interagency Collaborative Group on Environmental Carcinogenesis United States National Cancer Institute Bethesda, MD (1976)

(11) National Center for Toxicological Research Appendix B, Report of Chronic Studies Task Force Committee, April 13–21, 1972 National Center for Toxicological Research, Rockville, MD (1972)

(12) Organization for Economic Cooperation and Development Guidelines for Testing of Chemicals, Section 4-Health Effects, Part 451 Carcinogenicity Studies, Paris (1981)

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(15) Schwartz, E Toxicology of Neuroleptic Agents. *Industrial Pharmacology. Neuroleptics* S Fielding and H Lal Futura, Mt Kisco, NY pp 203–221 (1974)

(16) Sontag, J M et al Guidelines for Carcinogen Bioassay in Small Rodents NCI-CS-TR-1 United States Cancer Institute, Division of Cancer Control and Prevention, Carcinogenesis Bioassay Program Bethesda, MD

(17) Summary of the EPA Workshop on Carcinogenesis Bioassay via the Dermal Route EPA Report 50/6–89–002, 50/6–89–003 Washington, DC

(18) The Atlas of Dermal Lesions, EPA Report 20t–2004, U S Environmental Protection Agency, Washington, DC

(19) Toxicity and Clinical Trial Subcommittee, Committee on Safety of Medicine, November, 1977

(20) United States Environmental Protection Agency Office of Testing and Evaluation Proposed health effects test standards for toxic substances control act test rules 40 CFR Part 772 Standard for Development of Test Data. Subpart D Chronic Health Effects FEDERAL REGISTER No 91 (44 FR 27350–27362)

(21) United States Pharmaceutical Manufacturers Association Guidelines for the Assessment of Drug and Medical Device Safety in Animals (1977)

(22) World Health Organization (WHO) Guidelines for Evaluation of Drugs for Use in Man WHO Technical Report Series No. 563 (WHO), Geneva (1975)

(23) World Health Organization (WHO) Part I Environmental Health Criteria 6 *Principles and Methods for Evaluating the Toxicity of Chemicals* (WHO), Geneva (1978)

(24) World Health Organization (WHO) Principles for Pre-Clinical Testing of Drug Safety WHO Technical Report Series No 341 (WHO), Geneva (1966)

United States
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Prevention Pesticides
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(7101)

EPA 712–C–98–212
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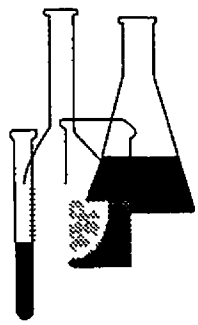
Health Effects Test Guidelines

OPPTS 870.4300
Combined Chronic Toxicity/Carcinogenicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S. Environmental Protection Agency under the Toxic Substances Control Act (15 U S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S C. 136, *et seq*)

Final Guideline Release: This guideline is available from the U S Government Printing Office, Washington, DC 20402 on disks or paper copies call (202) 512–0132 This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http //www epa gov/epahome/research htm) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines "

OPPTS 870 4300 Combined chronic toxicity/carcinogenicity.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 798 3320 Combined Chronic Toxicity/Oncogenicity, OPP 83-5 Combined Chronic Toxicity/Oncogenicity (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09-82-025, 1982, and OECD 453 Combined Chronic Toxicity/Carcinogenicity Studies

(b) **Purpose.** The objective of a combined chronic toxicity/carcinogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure The application of this guideline should generate data which identify the majority of chronic and carcinogenicity effects and determine dose-response relationships The design and conduct should allow for the detection of neoplastic effects and a determination of the carcinogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects

(c) **Definitions.** The definitions in section 3 of TSCA and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this guideline The following definitions also apply to this guideline

Carcinogenicity is the development of neoplastic lesions as a result of the repeated daily exposure of experimental animals to a chemical by the oral, dermal, or inhalation routes of exposure

Chronic toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral, dermal, or inhalation routes of exposure

Cumulative toxicity is the adverse effects of repeated dose occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissues

Dose in a combined chronic toxicity/carcinogenicity study is the amount of test substance administered via the oral, dermal, or inhalation routes for a period of up to 24 months Dose is expressed as weight of the test substance per unit body weight of test animal (milligrams per kilogram), or as weight of the test substance in parts per million (ppm) in food or drinking water When exposed via inhalation, dose is expressed as weight of the test substance per unit volume of air (milligrams per liter) or as parts per million per day For dermal application, dose is ex-

pressed as weight of the test substance (grams, milligrams) per unit body weight of the test animal (milligrams per kilogram) or as weight of the substance per unit surface area (milligrams per square centimeter) per day

No-observed-effect-level (NOEL) is the maximum dose used in a study which produces no observed adverse effects The NOEL is usually expressed in terms of the weight of a test substance given daily per unit weight of test animal (milligrams per kilogram per day)

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects or if toxic effects would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary

(e) **Test procedures—(1) Animal selection—(i) Species and strain.** Preliminary studies providing data on acute, subchronic, and metabolic responses should have been carried out to permit an appropriate choice of animals (species and strain) As discussed in other guidelines, the mouse and rat have been most widely used for assessment of carcinogenic potential, while the rat and dog have been most often studied for chronic toxicity For the combined chronic toxicity/carcinogenicity study via the oral and inhalation routes, the rat is the species of choice and for the dermal route, the mouse is species of choice If other species are used, the tester should provide justification/reasoning for their selection The strain selected should be susceptible to the carcinogenic or toxic effect of the class of substances being tested, if known, and provided it does not have a spontaneous background incidence too high for meaningful assessment Commonly used laboratory strains should be employed.

(ii) **Age/weight.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization

(B) Dosing should generally begin no later than 8 weeks of age

(C) At commencement of the study, the weight variation of animals used should be within 20 percent of the mean weight for each sex

(D) Studies using prenatal or neonatal animals may be recommended under special conditions

(iii) **Sex.** (A) Equal numbers of animals of each sex should be used at each dose level

(B) Females should be nulliparous and nonpregnant

(iv) **Numbers.** (A) At least 100 rodents (50 males and 50 females) should be used at each dose level and concurrent control group At least 20 additional rodents (10 males and 10 females) should be used for satellite dose groups and the satellite control group The purpose of the satellite group is to allow for the evaluation of chronic toxicity after 12 months of exposure to the test substance

(B) For a meaningful and valid statistical evaluation of long term exposure and for a valid interpretation of negative results, the number of animals in any group should not fall below 50 percent at 15 months in mice and 18 months in rats Survival in any group should not fall below 25 percent at 18 months in mice and 24 months in rats

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required.

(D) Each animal should be assigned a unique identification number Dead animals (and their preserved organs) and tissues, and microscopic slides should be identified by reference to the unique numbers assigned

(v) **Husbandry.** (A) Animals may be group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal The biological properties of the test substance or toxic effects (e g , morbidity, excitability) may indicate a need for individual caging Rodents should be housed individually in dermal studies and during exposure in inhalation studies

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C

(C) The relative humidity of the experimental animal rooms should be 50 ± 20 percent.

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark.

(E) Control and test animals should be fed from the same batch and lot The feed should be analyzed to assure uniform distribution and adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test Animals should be fed and watered ad libitum with food replaced at least weekly

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine An acclimation period of at least five days is recommended

(2) **Control and test substances** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle If a vehicle or dilu-

ent is needed, it should not elicit toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution in oil, and finally solution in other vehicles

(ii) One lot of the test substance should be used throughout the duration of the study if possible, and the research sample should be stored under conditions that maintain its purity and stability Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound, and, if possible, the name and quantities of contaminants and impurities

(iii) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture

(3) **Control groups.** A concurrent control group is required. This group should be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(4) **Dose levels and dose selection.** (i) For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects. A rationale for the doses selected must be provided

(ii) The highest dose level in rodents should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors The highest dose should be determined based on the findings from a 90–day study to ensure that the dose used is adequate to assess the chronic toxicity and the carcinogenic potential of the test substance Thus, the selection of the highest dose to be tested is dependent upon changes observed in several toxicological parameters in subchronic studies. The highest dose tested need not exceed 1,000 mg/kg/day

(iii) The intermediate-dose levels should be spaced to produce a gradation of toxic effects

(iv) The lowest-dose level should produce no evidence of toxicity

(v) For skin carcinogenicity studies, when toxicity to the skin is a determining factor, the highest dose selected should not destroy the functional integrity of the skin, the intermediate doses should be a minimally irritating dose and the low dose should be the highest nonirritating dose

(vi) The criteria for selecting the dose levels for skin carcinogenicity studies, based on gross and histopathologic dermal lesions, are as follows

(A) Gross criteria for reaching the high dose

(*1*) Erythema (moderate)

(*2*) Scaling

(*3*) Edema (mild)

(*4*) Alopecia

(*5*) Thickening

(B) Histologic criteria for reaching the high dose

(*1*) Epidermal hyperplasia

(*2*) Epidermal hyperkeratosis

(*3*) Epidermal parakeratosis

(*4*) Adnexal atrophy/hyperplasia

(*5*) Fibrosis

(*6*) Spongiosis (minimal-mild)

(*7*) Epidermal edema (minimal-mild)

(*8*) Dermal edema (minimal-moderate)

(*9*) Inflammation (moderate)

(C) Gross criteria for exceeding the high dose

(*1*) Ulcers-fissures, exudate/crust (eschar),nonviable (dead) tissues

(*2*) Anything leading to destruction of the functional integrity of the epidermis (e g , caking, fissuring, open sores, eschar)

(D) Histologic criteria for exceeding the high-dose

(*1*) Crust (interfollicular and follicular)

(*2*) Microulcer

(*3*) Degeneration/necrosis (mild to moderate)

(*4*) Epidermal edema (moderate to marked)

(*5*) Dermal edema (marked)

(*6*) Inflammation (marked)

(5) **Administration of the test substance.** The three main routes of administration are oral, dermal, and inhalation The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans

(i) **Oral studies.** If the test substance is administered by gavage, the animals are dosed with the test substance on a 7–day per week basis for a period of at least 18 months for mice and hamsters and 24 months for rats However, based primarily on practical considerations, dosing by gavage on a 5–day per week basis is acceptable If the test substance is administered in the drinking water or mixed in the diet, then exposure should be on a 7–day per week basis

(ii) **Dermal studies.** (A) Preparation of animal skin Shortly before testing, fur should be clipped from not less than 10 percent of the body surface area for application of the test substance In order to dose approximately 10 percent of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half way down the flank on each side of the animal should be shaved Shaving should be carried out approximately 24 hours before dosing Repeated clipping or shaving is usually needed at approximately weekly intervals When clipping or shaving the fur, care should be taken to avoid abrading the skin which could alter its permeability

(B) Preparation of test substance Liquid test substances are generally used undiluted, except as indicated in paragraph (e)(4)(vi) of this guideline Solids should be pulverized when possible The substance should be moistened sufficiently with water or, when necessary, with a suitable vehicle to ensure good contact with the skin When a vehicle is used, the influence of the vehicle on toxicity of, and penetration of the skin by, the test substance should be taken into account The volume of application should be kept constant, e g less than 100 µL for the mouse and less than 300 µL for the rat Different concentrations of test solution should be prepared for different dose levels

(C) Administration of test substance The duration of exposure should be at least 18 months for mice and hamsters and 24 months for rats. Ideally, the animals should be treated with test substance for at least 6 h/day on a 7-day per week basis However, based on practical considerations, application on a 5-day per week basis is acceptable. Dosing should be conducted at approximately the same time each day The test substance should be applied uniformly over the treatment site The surface area covered may be less for highly toxic substances As much of the area should

be covered with as thin and uniform a film as possible For rats, the test substance may be held in contact with the skin with a porous gauze dressing and nonirritating tape if necessary The test site should be further covered in a suitable manner to retain the gauze dressing plus test substance and to ensure that the animals cannot ingest the test substance The application site should not be covered when the mouse is the species of choice The test substance may be wiped from the skin after the 6-hour exposure period to prevent ingestion

(iii) **Inhalation studies.** (A) The animals should be exposed to the test substance, for 6 h/day on a 7–day per week basis, for a period of at least 18 months in mice and 24 months in rats However, based primarily on practical considerations, exposure for 6 h/day on a 5–day per week basis is acceptable

(B) The animals should be tested in dynamic inhalation equipment designed to sustain a minimum air flow of 10 air changes per hour, an adequate oxygen content of at least 19 percent, and uniform conditions throughout the exposure chamber Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding areas It is not normally necessary to measure chamber oxygen concentration if airflow is adequate

(C) The selection of a dynamic inhalation chamber should be appropriate for the test substance and test system Where a whole body chamber is used, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance To ensure stability of a chamber atmosphere, the total volume occupied by the test animals should not exceed 5 percent of the volume of the test chamber. It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order to minimize oral exposures due to animals licking compound off their fur The animals should be acclimated and heat stress minimized

(D) The temperature at which the test is performed should be maintained at 22 ± 2 °C The relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g , tests of aerosols, use of water vehicle) this may not be practicable

(E) The rate of air flow should be monitored continuously but recorded at least three times during the exposure

(F) Temperature and humidity should be monitored continuously but should be recorded at least every 30 minutes

(G) The actual concentrations of the test substance shall be measured in the animal's breathing zone During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable and monitored continuously or intermittently depending on the method of

analysis Chamber concentration may be measured using gravimetric or analytical methods as appropriate If trial run measurements are reasonably consistent (± 10 percent for liquid aerosol, gas, or vapor, ± 20 percent for dry aerosol), then two measurements should be sufficient If measurements are not consistent, three to four measurements should be taken Whenever the test substance is a formulation, or it is necessary to formulate the test substance with a vehicle for aerosol generation, the analytical concentration must be reported for the total formulation and not just for the active ingredient (AI) If, for example, a formulation contains 10 percent AI and 90 percent inerts, a chamber analytical limit concentration of 2 mg/L would consist of 0.2 mg/L of the AI It is not necessary to analyze inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation; the grounds for this conclusion must be provided in the study report If there is some difficulty in measuring chamber analytical concentration due to precipitation, nonhomogeneous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary

(H) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations with respect to particle size The mass median aerodynamic diameter (MMAD) particle size range should be between 1–3 µm The particle size of hygroscopic materials should be small enough when dry to assure that the size of the swollen particle will still be within the 1–3 µm range Measurements of aerodynamic particle size in the animal's breathing zone should be measured during a trial run If MMAD values for each exposure level are within 10 percent of each other, then two measurements during the exposures should be sufficient If pretest measurements are not within 10 percent of each other, three to four measurements should be taken

(I) Feed should be withheld during exposure Water may also be withheld during exposure

(6) **Observation period.** (i) This time period should not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice For longer time periods, and where any other species are used, consultation with the Agency in regard to the duration of the study is advised

(ii) Animals in a satellite group to assess chronic toxicity should be observed for 12 months

(7) **Observation of animals.** (i) Observations should be made at least twice each day for morbidity and mortality Appropriate actions should be taken to minimize loss of animals to the study (e g , necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals) General clinical observations should be made at least once a day, preferably at the same time each day, taking into consid-

eration the peak period of anticipated effects after dosing The clinical condition of the animal should be recorded

(ii) A careful clinical examination should be made at least once prior to the initiation of treatment (to allow for within subject comparisons) and once weekly during treatment in all animals These observations should be made outside the home cage, preferably in a standard arena, and at similar times on each occasion Effort should be made to ensure that variations in the observation conditions are minimal Observations should be detailed and carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e g , lacrimation, piloerection, pupil size, unusual respiratory pattern) Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e g , excessive grooming, repetitive circling) or bizarre behavior (e g , self-mutilation , walking backwards) should be recorded

(iii) Once, near the end of the first year of the exposure period and in any case not earlier than in month 11, assessment of motor activity, grip strength, and sensory reactivity to stimuli of different types (e g , visual, auditory, and proprioceptive stimuli) should be conducted in rodents Further details of the procedures that could be followed are described in the references listed under paragraphs (h)(2), (h)(7), (h)(9), (h)(12), (h)(13), and (h)(25) of this guideline

(iv) Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits

(v) Exceptionally, functional observations may be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with functional test performance

(vi) Body weights should be recorded individually for all animals once prior to administration of the test substance, once a week during the first 13 weeks of the study and at least once every 4 weeks thereafter unless signs of clinical toxicity suggest more frequent weighing to facilitate monitoring of health status

(vii) Measurements of feed consumption should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise Measurements of water consumption should be determined at the same intervals if the test material is administered in drinking water

(viii) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible At the end of the study period, all survivors should be sacrificed Animals in the satellite group should be sacrificed after 12 months of exposure to the test substance (interim sacrifice)

(8) **Clinical pathology.** Hematology, clinical chemistry and urinalyses should be performed from 10 animals per sex per group The parameters should be examined at approximately 6 month intervals during the first 12 months of the study If possible, these collections should be from the same animals at each interval If hematological and biochemical effects are seen in the subchronic study, testing should also be performed at 3 months Overnight fasting of animals prior to blood sampling is recommended

(i) Hematology The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time

(ii) Clinical chemistry (A) Parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity.

(B) The recommended clinical chemistry determinations are potassium, sodium, glucose, total cholesterol, urea nitrogen, creatinine, total protein, and albumin More than two hepatic enzymes, (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, or gamma glutamyl transpeptidase) should also be measMeasurements of additional enzymes (of hepatic or other origin) and bile acids, may also be useful

(iii) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated

(iv) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include calcium, phosphorus, fasting triglycerides, hormones, methemoglobin, and cholinesterases

(v) Urinalyses Urinalysis for rodents should be performed at the end of the first year of the study using timed urine collection Urinalysis determinations include appearance, volume, osmolality or specific gravity, pH, protein, glucose, and blood/blood cells

(9) **Ophthalmological examination.** Examinations should be made on all animals using an ophthalmoscope or an equivalent device prior to the administration of the test substance and at termination of the study on 10 animals per sex in the high-dose and control groups If changes in eyes are detected, all animals should be examined

(10) **Gross necropsy.** (i) A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in a moribund condition

(ii) At least, the liver, kidneys, adrenals, testes, epididymides, ovaries, *uterus, spleen, brain, and heart should be trimmed and weighed wet,* as soon as possible after dissection to avoid drying The lungs should be weighed if the test substance is administered by the inhalation route The organs should be weighed from interim sacrifice animals as well as from at least 10 animals per sex per group at terminal sacrifice

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder (when present)

(B) Nervous system—brain (multiple sections, including cerebrum, cerebellum and medulla/pons), pituitary, peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle), spinal cord (three levels, cervical, mid-thoracic, and lumbar), eyes (retina, optic nerve)

(C) Glandular system—adrenals, parathyroid, thyroid

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose

(E) Cardiovascular/Hematopoietic system—aorta, heart, bone marrow (and/or fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland

(G) Other—all gross lesions and masses, skin

(iv) In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved In dermal studies, skin from treated and adjacent control skin sites should be examined and preserved

(v) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues The proper inflation and fixation

of the lungs in inhalation studies is essential for appropriate and valid histopathological examination

(vi) Information from clinical pathology and other in-life data should be considered before microscopic examination, since these data may provide significant guidance to the pathologist

(12) **Histopathology.** (i) The following histopathology should be performed

(A) Full histopathology on the organs and tissues, listed under paragraph (e)(10)(iii) of this guideline of all animals in the control and high dose groups and of all animals that died or were killed during the study

(B) All gross lesions in all animals

(C) Target organs in all animals

(ii) If the results show substantial alteration of the animal's normal life span, the induction of effects that might affect a neoplastic response, or other effects that might compromise the significance of the data, the next lower levels should be examined fully as described in paragraph (e)(12)(i) of this guideline

(iii) An attempt should be made to correlate gross observations with microscopic findings

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming.

(f) **Data and reporting—(1) Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion

(ii) When applicable, all observed results, quantitative and qualitative, should be evaluated by an appropriate statistical method Any generally accepted statistical methods may be used, the statistical methods including significance criteria should be selected during the design of the study

(2) **Evaluation of study results.** (i) The findings of a combined chronic toxicity/carcinogenicity study should be evaluated in conjunction with the findings of previous studies and considered in terms of the toxic effects, the necropsy and histopathological findings The evaluation will include the relationship between the dose of the test substance and the presence incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight

changes effects on mortality and any other general or specific toxic effects

(ii) In any study which demonstrates an absence of toxic effects further investigation to establish absorption and bioavailablity of the test substance should be considered

(iii) In order for a negative test to be acceptable, it should meet the following criteria—no more than 10 percent of any group is lost due to autolysis, cannibalism, or management problems, and survival in each group is no less than 50 percent at 15 months for mice and 18 months for rats Survival should not fall below 25 percent at 18 months for mice and 24 months for rats

(iv) The use of historical control data from an appropriate time period from the same testing laboratory (i e, the incidence of tumors and other suspect lesions normally occurring under the same laboratory conditions and in the same strain of animals employed in the test) is helpful for assessing the significance of changes observed in the current study.

(3) **Test report.** (i) In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, 40 CFR part 160, and the OECD Principles of GLP (ISBN 92–64–12367–9), the following specific information should be reported

(A) Test substance characterization should include

(*1*) Chemical identification

(*2*) Lot or batch number

(*3*) Physical properties

(*4*) Purity/impurities

(*5*) Identification and composition of any vehicle used

(B) Test system should contain data on

(*1*) Species and strain of animals used and rationale for selection if other than that recommended

(*2*) Age including body weight data and sex

(*3*) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods

(*4*) Identification of animal diet

(*5*) Acclimation period

(C) Test procedure should include the following data

(*1*) Method of randomization used

(*2*) Full description of experimental design and procedure

(*3*) Dose regimen including levels, methods, and volume

(4) **Test results.** (i) Group animal data Tabulation of toxic response data by species, strain, sex, and exposure level for

(A) Number of animals exposed

(B) Number of animals showing signs of toxicity

(C) Number of animals dying

(ii) Individual animal data Data should be presented as summary (group mean) as well as for individual animals

(A) Time of death during the study or whether animals survived to termination

(B) Time of observation of each abnormal sign and its subsequent course

(C) Body weight data.

(D) Feed and water consumption data, when collected

(E) Achieved dose (milligrams/kilogram body weight) as a time-weighed average is the test substance is administered in the diet or drinking water

(F) Results of ophthalmological examination, when performed

(G) Results of hematological tests performed

(H) Results of clinical chemistry tests performed

(I) Results of urinalysis tests performed

(J) Results of observations made

(K) Necropsy findings including absolute/relative organ weight data.

(L) Detailed description of all histopathological findings

(M) Statistical treatment of results where appropriate

(N) Historical control data

(iii) In addition, for inhalation studies the following should be reported

(A) Test conditions The following exposure conditions must be reported

(*1*) Description of exposure apparatus including design, type, dimensions, source of air system for generating particulates and aerosols, method of conditioning air treatment of exhaust air and the method of housing the animals in a test chamber

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described

(B) Exposure data These should be tabulated and presented with mean values and a measure of variability (e g standard deviation) and should include

(*1*) Airflow rates through the inhalation equipment

(*2*) Temperature and humidity of air

(*3*) Actual (analytical or gravimetric) concentration in the breathing zone

(*4*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air)

(*5*) Particle size distribution, and calculated mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD)

(*6*) Explanation as to why the desired chamber concentration and/or particle size could not be achieved (if applicable) and the efforts taken to comply with this aspect of the guidelines

(g) **Quality assurance.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment The study must be conducted in compliance with the GLP regulations as described by the Agency (40 CFR parts 160 and 792) and the OECD Principles of GLP (ISBN 92–64–12367–9)

(h) **References.** The following references should be consulted for additional background information on this guideline

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